Universidad Autónoma de Madrid

Departamento de Biología Molecular

Tesis Doctoral

"The function of the proto-oncogene *c-myc* in B lymphocyte differentiation"

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Introduction

1. Introduction

1.1 The c-Myc transcription factor

The *c-myc* proto-oncogene, which encodes the c-Myc transcription factor, was originally identified as the human homologue to the viral oncogene (*v-myc*) of the avian myelocytomatosis retrovirus more than 25 years ago (Vennstrom et al., 1982). Since its discovery, it has been implicated in a variety of human malignancies. c-Myc is in fact one of the most frequently activated oncogenes, and is estimated to be involved in 20% of all human cancers, affecting about 100,000 US cancer deaths each year (Dang, 1999) (Nesbit et al., 1999). Due to its role in basic biological processes and its implications in tumorigenesis, the *c-myc* proto-oncogene has been the subject of intense study since its discovery, with more than 10,000 publications currently listed in the biomedical research literature.

1.1.1. c-Myc protein

The c-Myc protein belongs to the basic-helix-loop-helix leucine zipper (HLHZip) family of transcription factors. The Myc transcription factor family also comprises N-, L-, B-, and s-Myc; nonetheless, only c-, N-, and L-Myc are found in mammals. c-Myc is expressed ubiquitously during embryogenesis and in highly proliferative adult tissues. c-Myc protein consists of an N-terminal transactivation domain (TAD; required for transcriptional activation), a central region and a C-terminal domain (CTD; which is critical for DNA binding and protein interactions) (Fig. I1). c-Myc has four conserved regions known as Myc boxes (MB) that are essential for a variety functions. MBI and MBII are found in the TAD, while MBIII and MBIV are located in the central region (Fig. I1). MBI is necessary for gene

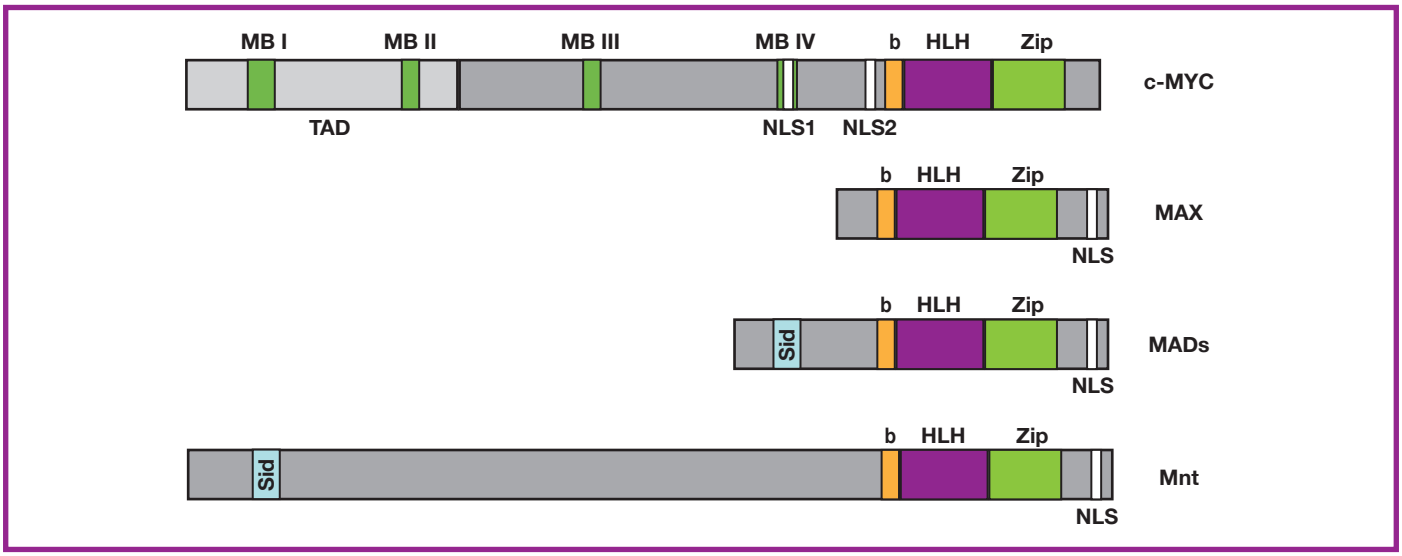

Fig. I1. Structure of Myc and Max transcription factors. Diagram of the c-Myc and Max proteins. For c-Myc, the scheme details the location of the transactivation domain (TAD) containing the conserved "Myc boxes" I and II (MBI and MBII), the central region that harbours MBIII and MBIV, and the nuclear localisation signals 1 and 2 (NLS1 and NLS2). The C-terminal domain contains the basic region (b) and the helix-loop-helix leucine zipper (HLH-Zip) motif that is necessary for dimerisation with its obligate partner Max, and subsequent DNA binding of Myc-Max heterodimers. All members of the network share the b-HLH-Zip motif. Sid: mSin3-interaction domain.

activation and protein degradation; MBII is critical for the majority of Myc's biological functions (Oster et al., 2002) (Pelengaris et al., 2002) (see later); MBIII is involved in transcriptional repression, apoptosis, transformation and lymphomagenesis (Herbst et al., 2005), and MBIV contributes to apoptosis, transformation and modulation of DNA-binding (Cowling et al., 2006). Small deletions or mutations in any of these MB are sufficient to affect Myc-dependent transactivation of target genes (Cowling and Cole, 2006). In addition, the CTD contains the basic region (b) and the HLHZip domain. The basic region is necessary for DNA binding through the consensus E-box sequence CACGTG, whereas the HLHZip domain is required for heterodimerisation with Max, the obligate heterodimerisation partner of Myc (see below).

1.1.2. Regulation of c-Myc protein

The regulation of c-Myc protein is controlled by the ubiquitin/26S proteasome pathway. c-Myc is a dynamically regulated phosphoprotein with at least 10 major phosphorylation sites spanning its length (Henriksson et al., 1993). Phosphorylation of c-Myc has an important role in the transcriptional modification, biological function and proteolysis of the Myc proteins. Two main phosphorylation sites are responsible for c-Myc stabilization and degradation, Ser62 and Thr58. Phosphorylation of Ser62 stabilises c-Myc, whereas phosphorylation of Thr58 leads to its degradation (Hann, 2006). Several studies indicate that Ser62 phosphorylation might be necessary for c-Myc oncogenic activity, while loss of Thr58 phosphorylation enhances tumorigenesis (Hann, 2006). c-Myc is a short-lived protein, with a half-life of 20-30 minutes (Hann and Eisenman, 1984); disruption of proteolysis of c-Myc protein could contribute to tumorigenesis. In fact, a prolonged c-Myc half-life has been found in a glioma cell line (Shindo et al., 1993) and during differentiation of an erythroleukemia cell line (Spotts and Hann, 1990).

1.1.3. The Myc/Max/Mad network of transcriptional regulators

c-Myc protein binds to E-boxes and transactivates transcription of its target genes by forming a heterodimer to another bHLHZip protein known as Max (Blackwood and Eisenman, 1991). Max is a small, ubiquitously expressed protein that binds to a whole collection of bHLHZip proteins (Baudino and Cleveland, 2001). c-Myc and Max form heterodimers through the HLHZip domain and contact DNA through the basic region (Fig. I1). The Max protein lacks a TAD; consequently, Myc is the heterodimer partner responsible for activation of the transcription of its target genes (Luscher and Larsson, 1999). Max can also form heterodimers with bHLHZip proteins of the Mad/Mxi/Mnt family; these complexes bind the same core consensus sequence as Myc-Max dimers, but act instead as transcriptional repressors (Amati et al., 2001). All these heterodimers are found *in vivo,* but Myc-Max complexes are predominant in proliferating cells, whereas Mad-Max and Mnt-Max complexes predominate in resting or differentiated cells (Ayer and Eisenman, 1993). The working model for this network is therefore based on the formation of Myc-Max heterodimers that bind to E boxes in the promoter regions of the specific target genes to activate their transcription. Once Myc executes its biological functions, it is rapidly degraded, and the pathway switches to a repressive state of transcription where Mad-Max dimers form, bind the same E boxes previously occupied by Myc-Max heterodimers, and repress target gene transcription (Amati et al., 2001; Knoepfler, 2007).

1.1.4. Transcriptional activation

The expression of the majority of genes in eukaryotic cells is mediated by RNA polymerase II (polII). DNA is normally tightly wound around histones; consequently, chromatin remodelling is required to allow transcription factors to access the promoter regions of genes. For c-Myc protein, at least two mechanisms of chromatin remodelling have been described so far, histone acetylation and ATP-dependent remodelling.

In the first mechanism, c-Myc recruits a co-activator complex containing transformation/transcription domain-associated protein (TRRAP) and histone acetyl transferases (HAT) such as GNC5 (McMahon et al., 1998; McMahon et al., 2000) and TIP60 (Frank et al.,

2003). The HAT acetylate NH_2-terminal lysines of histones located near the transcription start site (Jenuwein and Allis, 2001). Acetylation opens up the chromatin structure, allowing enhanced transcription of Myc-specific target genes. In the second mechanism, ATP-dependent chromatin remodelling, Myc interacts with INI1/hSNF5 (Cheng et al., 1999), a component of the SWI-SNF complex that remodels chromatin in an ATP-dependent manner. ATP-dependent reorganization of the DNA induces nucleosomes to move smoothly along the DNA, making it more accessible (Whitehouse et al., 1999) (Fig. I3, a). A recent study suggests that c-Myc might regulate chromatin structure in a general fashion (Knoepfler et al., 2006). As outlined above, TRRAP mediates c-Myc binding to HAT TIP60 and hGCN5, which acetylate H4 and H3, respectively. This study shows that Myc controls the genome-wide levels of H3 and H4 modification. These global changes in chromatin structure may account for the extremely large number of Myc target genes that have been identified so far –in fact, Myc targets constitute nearly 10% of all cellular genes (Cowling and Cole, 2006).

1.1.5. Transcriptional repression

Whereas the mechanisms by which c-Myc activates transcription of its target genes is well understood, less is known about how Myc represses

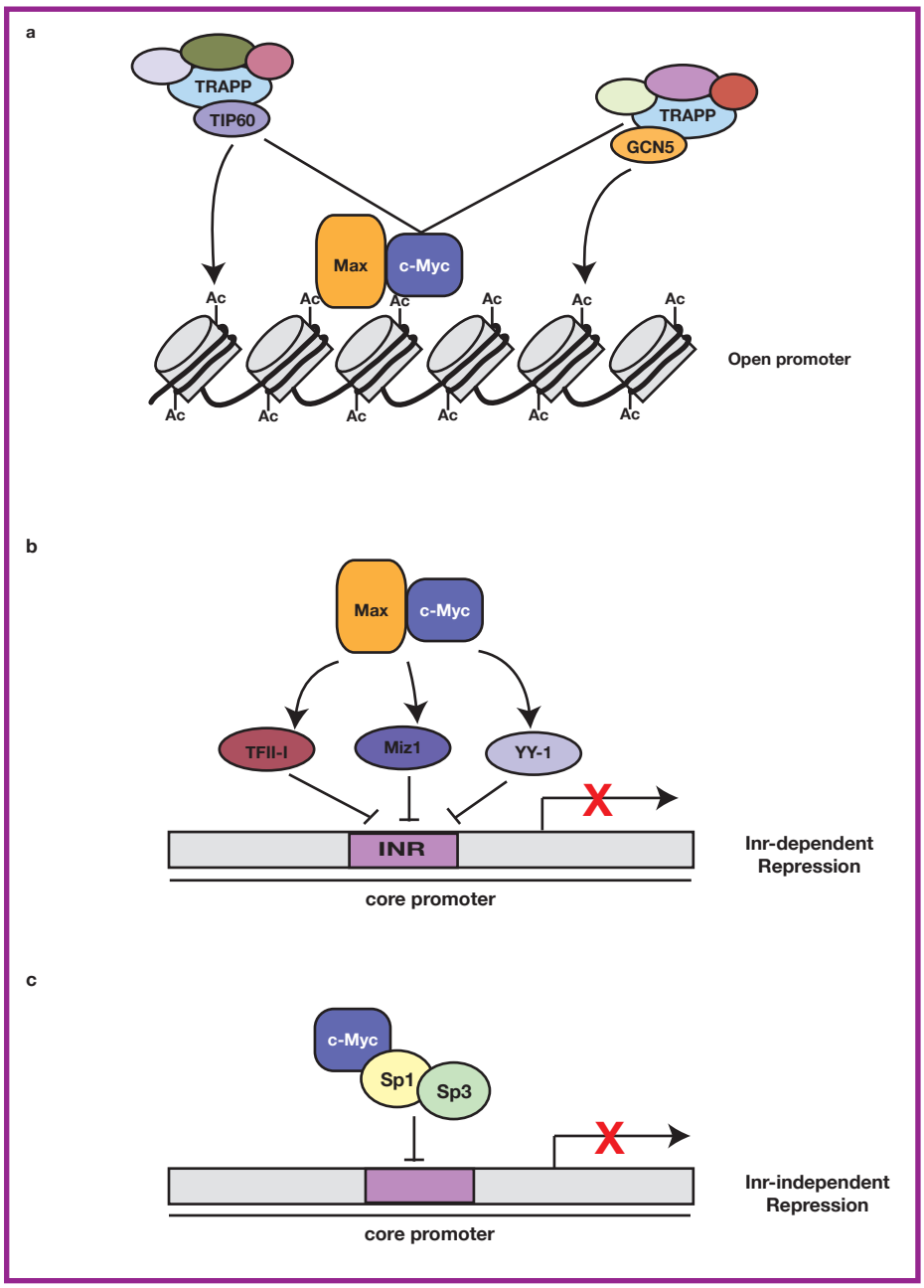

Fig. I2. Mechanisms of transcriptional activation and repression by c-Myc. (a) Myc mediates transcriptional activation by increasing local histone acetylation at promoters of its target genes. Myc recruits acetyltransferase complexes that contain TRAPP and either GCN5 or Tip60. These complexes alter the acetylation of nucleosomes, and can also recruit other factors that activate transcription, such as the SWI/SNF chromatin-remodelling complex. (b, c) At least two mechanisms of transcriptional repression have been described; in one, (b) Inr-dependent repression involves the specific binding and inhibition of the transcriptional activators TFII-I, Miz1 and YY-1 at Inr-containing promoters. In the other, (c) Myc represses transcription of p15, p21, and gadd45 by a process that involves binding to Sp1 and/or Sp3, thereby inhibiting their transcriptional activity.

transcription. Most target genes that are repressed by c-Myc are TATA-less promoters that contain initiator (Inr) elements within their promoter regions; however, c-Myc can also mediate repression of genes that lack Inr elements (Gartel and Shchors, 2003). In the case of Inr-dependent repression, c-Myc interacts with transcriptional activators that bind to Inr such as TFII-I, YY1 and Miz-1, leading to inhibition of target gene activation (Henriksson and Luscher, 1996) (Fig. I3, b). It was recently shown that a conserved element in MBIII is important for transcriptional repression (Kurland and Tansey, 2008). Myc represses transcription of target genes Id2 and Gadd153 by a process that includes histone deacetylation, a mechanism that involves MBIII recruitment of the histone deacetylase HDAC3 to the Id2 and Gadd153 promoters. In an Inr-independent mechanism, c-Myc also represses transcription of genes that lack an Inr element in their promoter region. Examples of genes repressed in this way are *gadd45* (Marhin et al., 1997), *p15* (Feng et al., 2002) and $p21^{Cip1}$ (Gartel et al., 2001). It is thought that the mechanism by which c-Myc represses these genes is by binding to transcription factors Sp1 and Sp1/Sp3 via the c-Myc central region, thereby inhibiting its transcriptional activity (Gartel and Shchors, 2003) (Fig. I3, c).

1.2. The biological functions of c-Myc

The expression of c-Myc is elevated during embryogenesis and in adult tissues with a high proliferation rate, such as skin and gut. c-Myc exerts its biological functions through its ability to bind to target genes and either activate or repress their transcription. In recent years, several groups have used a variety of techniques to identify potential target genes. Fig I3 summarises

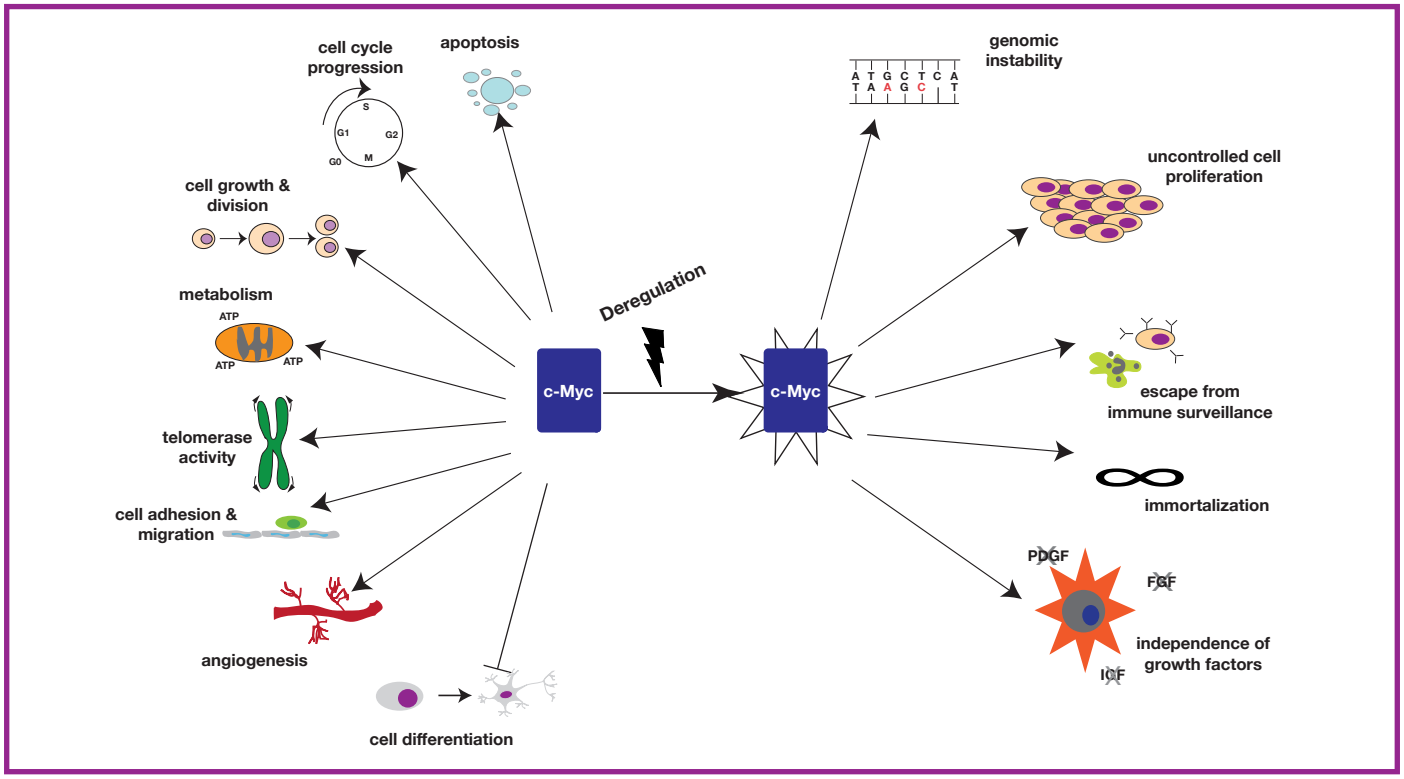

Fig. I3. c-Myc biological functions: cell processes controlled by c-Myc during normal conditions and tumorigenesis. Myc regulates basic cell processes such as growth and division (by regulating chromatin modifications and components of the biosynthetic machinery), cell cycle progression (see text), apoptosis (see text), differentiation (by downregulating growth arrest genes), cell metabolism (by modulating the expression of genes involved in glycolysis, amino acid biosynthesis and transport, synthesis of macromolecules and DNA metabolism), angiogenesis (by upregulation of VEGF), cell adhesion and motility (by controlling integrin expression). Deregulation of c-Myc expression can result in genomic instability, uncontrolled cell proliferation, escape from immune surveillance, immortalization and growth factor inhibition

the cellular processes controlled by Myc during normal conditions and tumorigenesis.

1.2.1. c-Myc in cell proliferation

c-Myc protein levels are highly regulated throughout the cell cycle; changes depend on the phase of the cycle in which the cell is found. In quiescent cells *in vitro*, c-Myc expression is barely detectable. After mitogenic or serum stimulation, c-Myc levels are rapidly induced and cells enter the G1 phase of the cell cycle. mRNA levels decline thereafter to low, but detectable, levels in proliferating cells. If serum or growth factors are removed, c-Myc levels decline to undetectable levels and the cycle is arrested. Numerous studies have shown that c-Myc regulates the cell cycle through its ability to activate or repress the transcription of genes involved in cell cycle progression. c-Myc regulates the G1/S checkpoint, and c-Myc expression is reportedly sufficient to overcome the checkpoint and to induce S phase entry in the absence of growth factors (Eilers, 1999). Studies of Rat1a *c-myc*$^{-/-}$ cells and in c-Myc-deficient mouse embryonic fibroblasts (MEF) showed that these cells have both prolonged doubling times and G1 and G2 phases (de Alboran et al., 2001; Mateyak et al., 1997).

Cell cycle progression is controlled by the activities of the cyclin-dependent kinases (CDK) and the cyclin-dependent kinase inhibitors (CDKI). c-Myc activates transcription of genes involved in cell cycle progression such as *cyclin D1, D2, E* and *A* (Bouchard et al., 1999; Daksis JI, 1994; Hoang AT et al., 1994), as well as *cdk4* (Hermeking et al., 2000) and *cdc25a* (Galaktionov et al., 1996). c-Myc also represses transcription of cyclin kinase inhibitors such as $p15^{ink4b}$, $p21^{cip1}$ and $p27^{kip1}$ (Dang, 1999; Grandori et al., 2000).

1.2.2. c-Myc–induced apoptosis

The first evidence that suggested a role for c-Myc in apoptosis derived from the observation that ectopic c-Myc expression in fibroblasts in the absence of survival factors led to apoptosis and loss of the entire population (Evan et al., 1992); indeed, subsequent studies identified the involvement of many Myc target genes in apoptosis. c-Myc induces apoptosis by at least four different mechanisms. The first of these is by inducing cytochrome c release from mitochondria; cytochrome c associates with APAF1 protein, activating pro-caspase 9, which in turn activates the downstream caspase cascade and leads to apoptosis (Juin et al., 1999). In a second mechanism, apoptosis is induced by activation of BAX, a pro-apoptotic molecule that also induces cytochrome c release from mitochondria (Soucie et al., 2001). A third pathway is by enhancing cell sensitivity to signalling through the CD95 death receptor, which triggers association of the intracellular adaptor protein FADD with the CD95 receptor; FADD recruits pro-caspase 8, leading to activation of the caspase cascade (Hueber et al., 1997). Finally, c-Myc induces apoptosis by indirect activation of p53 via ARF, which activates the p19/Mdm2/p53 apoptotic pathway (Zindy et al., 1998) (Fig. I4).

The variety of c-Myc-induced apoptosis routes suggests that the mechanism chosen might be determined by several factors including cell type, tissue location and the presence or absence of additional mutations in other pro- and anti-apoptotic genes (Pelengaris et al., 2002).

1.2.3. c-Myc and cell growth

An important aspect of proliferation is the capacity of the cell to increase in size and to coordinate this growth with division (Neufeld and Edgar, 1998). The means by which c-Myc controls cell growth is not fully understood, but it was shown that c-Myc is able to influence cell growth by binding to elements in the promoters of the rate-limiting translation initiator factors eIF4E and eIF2α (Jones et al., 1996; Rosenwald et al., 1993). In addition, c-Myc-deficient cells have decreased protein and ribosomal RNA synthesis rates, which may result in reduced cell growth (Mateyak et al., 1997)

In *Drosophila*, c-Myc orthologue (*dmyc)* function is involved in cell size regulation. Downregulation of *dmyc* expression gives rise to smaller flies that develop normally, as a result of a decrease in cell size and number; on the contrary, *dmyc* overexpression yield larger cells with a normal division rate (Johnston et al., 1999). These results suggest that the effects of c-Myc on cell growth

are independent of those on cell division. In mammals, c-Myc overexpression in B lymphocytes in *Eμ-c-myc* transgenic mice produced an increase in cell size in the absence of cell cycle progression (Iritani and Eisenman, 1999). In addition, *c-myc* inactivation in liver causes a decrease in hepatocyte size as well as smaller cell area (Baena et al., 2005).

Recent reports show that c-Myc also regulates the transcription of genes that are transcribed by RNA polymerase III (such as tRNA and 5S rRNA) and RNA polymerase I (genes encoding ribosomal RNA) (Arabi et al., 2005; Grandori et al., 2005). Studies of c-Myc-mediated transcription of RNA polymerase III-transcribed genes suggest that this process is Max-independent and therefore E-box-independent. Consequently, c-Myc might act as a co-activator, rather than by binding to its specific DNA binding site (Gomez-Roman et al., 2003).

1.2.4. c-Myc and cell differentiation

Another important biological function of c-Myc is its role in cell differentiation. c-Myc is expressed in immature proliferating cells, while its antagonists, the Mad proteins, are normally expressed in non-proliferating and differentiated cells (Chin et al., 1995). Downregulation of *c-myc* expression is reported to trigger cell differentiation (Chang et al., 2000; Henriksson and Luscher, 1996), whereas ectopic *c-myc* expression blocks terminal differentiation in several cell types, both *in vivo* and *in vitro* (Facchini LM and LZ, 1998; Iritani and Eisenman, 1999). Some examples of c-Myc regulation of cell differentiation are provided by the transcription

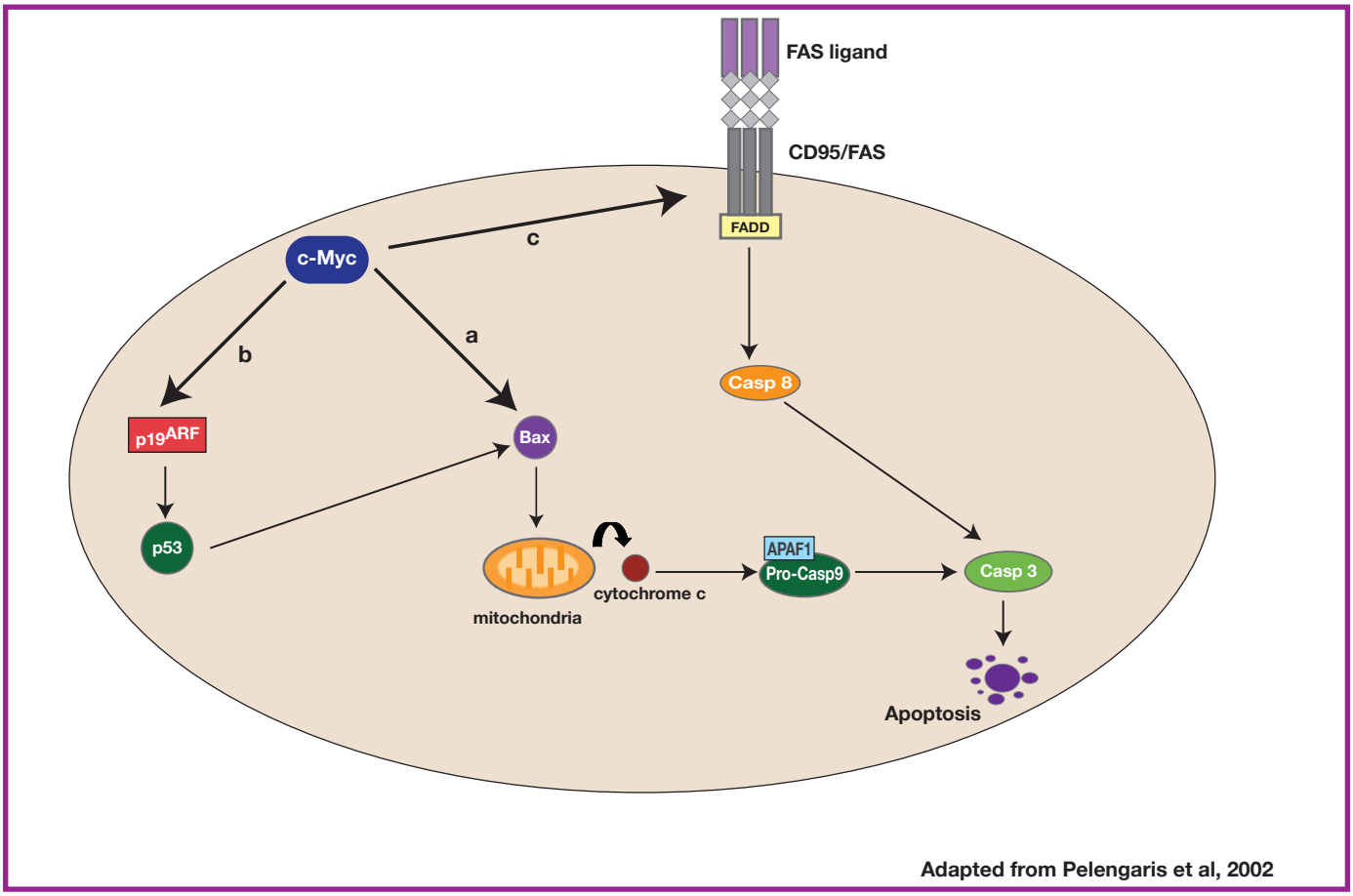

Fig. I4. c-Myc and apoptosis: different pathways of c-Myc-induced apoptosis. (a) c-Myc can activate Bax, which triggers cytochrome c release from mitochondria; cytochrome c associates with APAF-1 and pro-caspase9 and activates the downstream caspase cascade that leads to apoptosis. (b) c-Myc can also activate the tumour suppressor p53 via ARF, resulting in Bax transcription. (c) Ligation of death receptor CD95/Fas triggers association of the intracellular adaptor protein FADD with the CD95 receptor. FADD then recruits pro-caspase-8, activating the caspase cascade.

factor C/EBPα and the B lymphocyte-induced maturation protein-I (BLIMP-I). C/EBPα is necessary for myoblast commitment to the granulocytic lineage; it carries out the differentiation program by forming a complex with E2F/pRB that represses c-Myc expression (Johansen et al., 2001). In the case of B lymphocytes, BLIMP-I overexpression in a pro-monocytic cell line results in transcriptional repression of *c-myc*, which induces differentiation to macrophages and B cells (Chang et al., 2000). Overexpression of *c-myc* in *E*μ-*c-myc* mice leads to accumulation of immature pre-B cells in the bone marrow of these animals (Iritani and Eisenman, 1999).

The aim of this thesis is to study the role of the proto-oncogene *c-myc* in B cell differentiation. The next section summarises the basic concept of this developmental process.

1.3. B cell development

B cells are generated from pluripotent progenitors in a highly regulated process that takes place in foetal liver and in the adult bone marrow (BM), and further proceeds in the secondary lymphoid organs. B cell development progresses through differentiation several stages that can be followed by the expression of cell surface markers and the rearrangement of the immunoglobulin heavy (IgH) and light (IgL) chain genes. Mouse bone marrow contains B lineage cells at all developmental stages, from earliest progenitors to mature B cells. Various approaches have been used to identify and isolate cells at distinct developmental stages based on cell surface phenotype, which has led to several different nomenclatures that are summarised in table I1.

The earliest differentiated progenitors from HSC (haematopoietic stem cells) with dual lymphoid and myeloid potential are LMPP (lymphoid-primed multipotent progenitors) (Adolfsson et al., 2005). This population contains ELP (early lymphoid progenitors) with lymphoid-restricted lineage potential (Igarashi et al., 2002); these are the precursors of CLP (common lymphoid progenitors), which can give rise to B, T, dendritic or natural killer (NK) cells (Kondo et al., 1997). B lineage-restricted cells are identified by surface expression of B220, and arise from CLP. The first B lineage-committed cells, Fraction A (Fr. A) or pre-pro-B cells, are characterised by dual expression of the B220 and CD43 cell surface markers, and have little or no IgH rearrangement. These cells proceed to Fraction B/C cells (Fr. B/C) or pro-B cells, characterised by CD19 surface marker expression and by the onset of IgH chain rearrangement. IgH rearrangement takes place in a sequential fashion; first, a productive D_H-to-J_H joining is formed, followed by V_H-to-DJ_H rearrangement. Productive V_H-to-DJ_H joining

Phenotypic subset	Fr. A	Fr. B/C	Fr. C'	Fr. D	Fr. E
Philadelphia nomenclature	Pre-Pro-B	Pro-B	Early Pre-B	Late Pre-B	New-B
Basel nomenclature	Pro-B	Pre-B-I	Pre-B-I	Pre-B-II	
IgH	GL	rearranging	VDJ	VDJ	VDJ
IgL	GL	GL	GL	rearranging	VJ

Table I1. B cell nomenclature. B cell nomenclatures and their correspondence to Hardy fractions A to E, as well as their IgH and IgL rearrangement status.

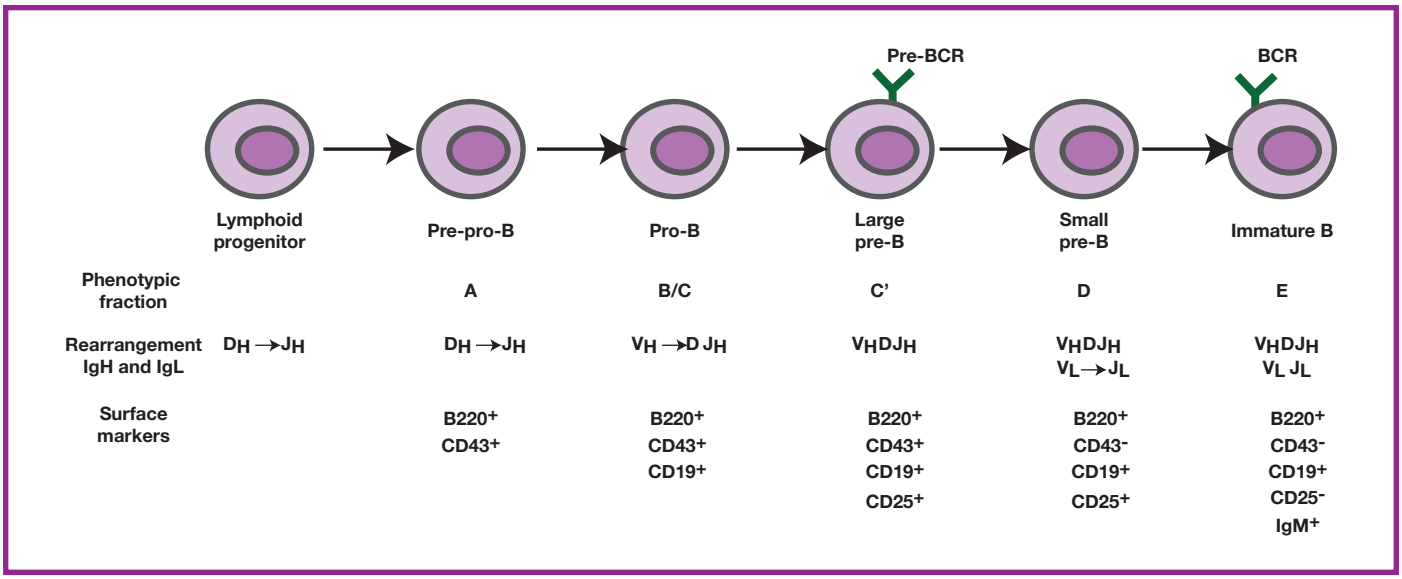

Fig. I5. B cell developmental pathway. Schematic representation of B cell development from lymphoid progenitors to immature B cells in the BM, showing Ig chain rearrangement status as well as the main surface markers used to discriminate the various developmental stages.

leads to expression of the μ chain, the hallmark of the pre-B cell stage, which comprises fraction C' (Fr. C') and D (Fr. D) cells. A fraction of the μH chain is expressed at the cell surface of large pre-B or Fr. C' cells, where it forms the pre-B cell receptor (pre-BCR) by pairing with the surrogate light chain consisting of λ5 and VpreB, and with the signalling molecules Igα and Igβ. Pre-BCR expression on the surface of large pre-B cells results in a proliferation burst in which large pre-B cells divide further before giving rise to small pre-B or Fr. D cells, where IgL chain rearrangements take place. Once a light chain gene is assembled and a complete IgM molecule is expressed on the cell surface, the cell becomes an immature B cell or Fraction E (Fr. E) cell (Fig. I5).

All the developmental stages explained above take place in BM and are antigen independent, but at this point immature B cells migrate into the periphery, where they are subject to selection for self-tolerance and ability to survive. Surviving cells undergo further differentiation to become mature B cells or Fraction F (Fr. F) cells, characterised by surface IgM and IgD expression; these are also known as naïve B cells and recirculate through secondary lymphoid organs until they encounter their specific antigen and are activated.

1.4. Transcriptional control of early B cell development

Early B cell development in the BM of mice is tightly controlled by a network of transcription factors with specific roles in the transition from one developmental stage to the next. Study of the genetic control of B cell development has been facilitated by the availability of mice bearing targeted mutations in genes encoding B cell-specific transcription factors. In this way, at least five transcription factors were identified as essential for B cell development, since their absence leads to a blockade in differentiation either at the initiation of B lineage commitment or at the onset of V_H-to-DJ_H recombination. Ikaros and PU.1 are required for the formation of the more primitive lymphoid progenitors (Medina et al., 2004; Nutt and Kee, 2007); E2A and EBF (Medina et al., 2004) are important at the earliest stages of B cell differentiation, and Pax5 is important in the lineage commitment in developing B lineage cells (Nutt et al., 1999) (Fig. I6).

1.4.1 PU.1

The *Sfpi1* gene encodes PU.1, a member of the ETS family of transcription factors, which is needed for the development of myeloid and lymphoid cell lineages.

PU.1 is considered an important transcription factor for the myeloid versus lymphoid lineage specification, since low PU.1 expression levels in haematopoietic precursors promote lymphoid development, whereas high levels promote myeloid development (DeKoter and Singh, 2000). PU.1-deficient mice die during foetal development at gestation day 18.5, and lack B, T, and myeloid progenitors (Scott et al., 1994). Foetal livers of PU.1$^{-/-}$ mice have reduced numbers of progenitors with dual lymphoid-myeloid potential; these cells fail to proliferate and differentiate into pro-B cells when cultured *in vitro* with stromal cells and IL-7 (Scott et al., 1997), as they do not express surface IL7-Rα chain and cannot respond to IL-7 signals (DeKoter et al., 2002). PU.1-deficient haematopoietic progenitors express neither the genes that encode the pre-BCR proteins (Igα, Igβ, λ5 and VpreB) nor the proteins Rag1, Rag2 or Pax5; they do express Ikaros and E2A, as well as EBF at very low levels (DeKoter et al., 2002). Retroviral transduction of IL7-Rα into PU.1$^{-/-}$ progenitors can restore IL-7-dependent proliferation and differentiation in pro-B cells, but at low frequency. It was recently shown that PU.1$^{-/-}$ fetal liver cells can give rise to B lineage cells when they are cultured on a stromal cell line and in the presence of stem cell factor (SCF), Flt3 ligand (Flt3L) and IL-7. In these conditions, B cell colonies are nonetheless generated at low efficiency and with delayed kinetics compared to wild-type cells. These results suggest that PU.1 is not necessary for B cell development (Ye et al., 2005).

The generation of PU.1 conditional mutant mice and mice with a hypomorphic allele of *Sfpi1* allowed study of the role of PU.1 in adult mice. Conditional PU.1$^{-/-}$ mice have impaired haematopoiesis and excess granulopoiesis, do not have CLP and, in competitive reconstitution assays, they are unable to generate lymphoid cells (Dakic et al., 2005). Moreover, when PU.1 is deleted in CLP or in B lineage cells, there is no effect on B cell development (Iwasaki et al., 2005; Polli et al., 2005). The mice with a hypomorphic *Sfpi1* allele show a block in B cell development from earliest precursors (Houston et al., 2007; Rosenbauer et al., 2006). These animal models suggest that PU.1 is as an important factor for lymphoid specification, but is dispensable for B cell differentiation.

1.4.2. Ikaros

The Ikaros gene (*Ikzf1)* encodes a number of Krüppel-type zinc finger proteins that are generated by alternative splicing (Molnar and Georgopoulos, 1994) and can act as transcriptional activators or repressors, depending on cell context. The Ikaros gene is widely expressed throughout the haematopoietic system, including haematopoietic stem cells and their committed progenitors (Kelley et al., 1998). Ikaros expression increases in developing lymphocytes and is maintained in granulocytes, but is downregulated in the macrophage and erythrocyte lineages (Kelley et al., 1998; Klug et al., 1998). Two mouse models have allowed study of the role of Ikaros in the transcriptional control of B cell development. Ikaros-deficient mice, or mice that express a dominant negative form of Ikaros, totally lack B, NK cells and foetal T cells; however, a small number of early T cell precursors are found in the thymus, and mature T cells are exported to the periphery (Georgopoulos, 2002). These mice do have LMPP; these cells, which are the precursors of CLP, retain lymphoid-myeloid differentiation potential (Adolfsson et al., 2005). When Ikaros-deficient LMPP are cultured *in vitro* in lymphocyte-promoting conditions, they cannot give rise to B cells; compared to WT LMPP, they generate T cells at reduced frequency and with slower kinetics, and also generate myeloid lineage cells (Yoshida et al., 2006). These LMPP have reduced Flt3, IL7Rα and Rag1 expression, which could explain the impairment in promoting lymphoid development (Yoshida et al., 2006). These studies therefore indicate that Ikaros is necessary for initiation of the lymphoid program, but not for LMPP generation.

A recent paper demonstrates an essential role for Ikaros in regulating B cell fate commitment and IgH chain rearrangements. Restored EBF expression in Ikaros-deficient hematopoietic progenitors rescues B cell development in these cells, giving rise to Ikaros-deficient pro-B cells (Reynaud et al., 2008). These cells express EBF and Pax5, but in the absence of Ikaros, they differentiate into macrophages. Ikaros also controls IgH chain rearrangements at the pro-B cell stage, as it regulates Rag1 and Rag2 expression and compaction of the IgH locus. This study suggests that Ikaros promotes

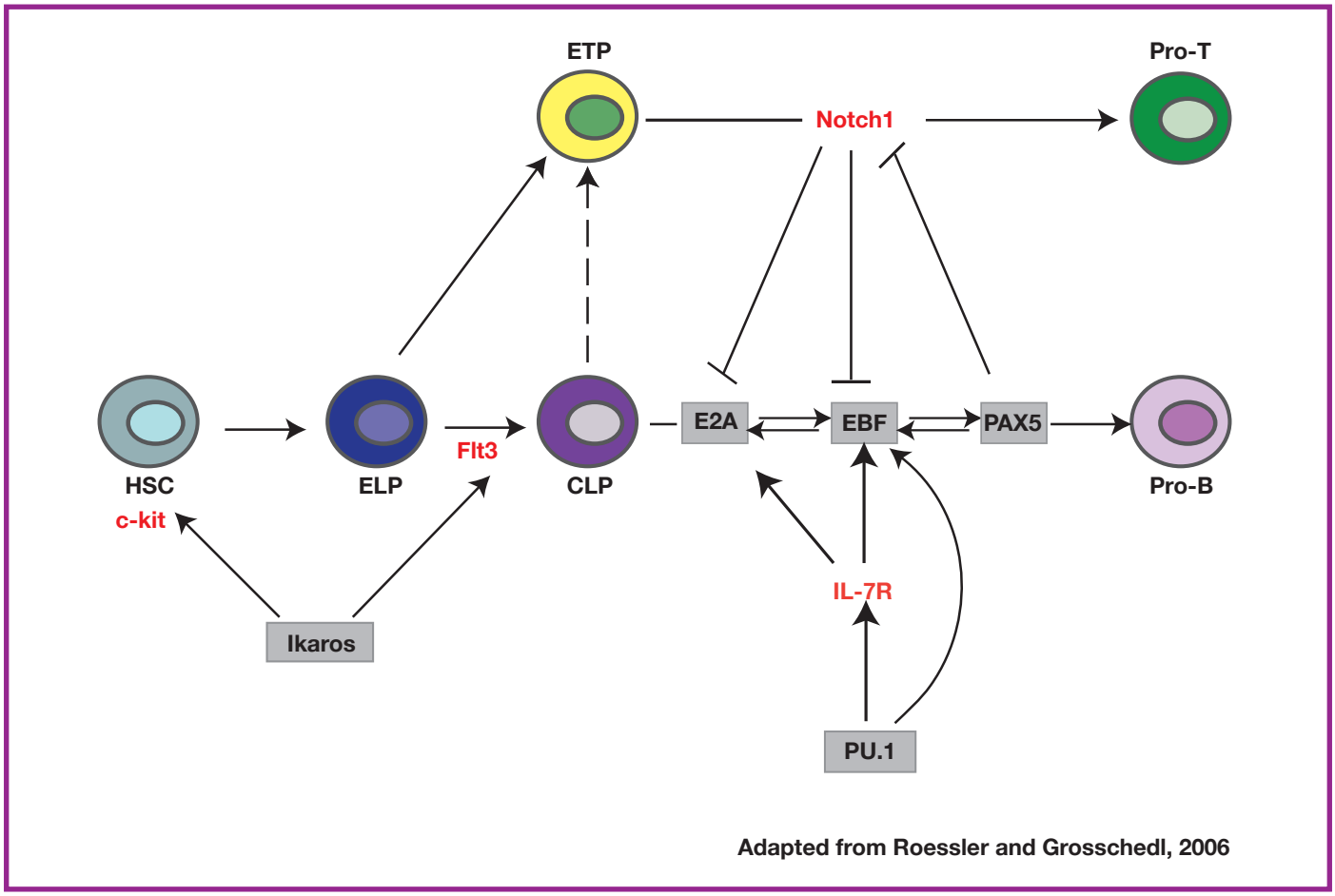

Fig. I6. Transcription factors regulating early B cell development. The network of transcription factors and signalling components known to regulate initiation of the lymphoid program, lineage specification and commitment. The scheme represents the stage at which the absence of these factors leads to a developmental block. Signalling pathways important in B cell differentiation are shown in red. HSC, haematopoietic stem cell; ELP, early lymphoid progenitor; CLP, common lymphoid progenitor; ETP, early T cell progenitor.

B cell identity by repressing alternative lineage myeloid genes and by inducing recombination events at the Igh locus.

1.4.3. E2A

Tcfe2a encodes two splice variants, E12 and E47, which are members of the bHLH family of transcription factors (Kadesch, 1992). With E2A, two related proteins (E2-2 and HEB) form the E protein family. Of these, E2A is found predominantly in B lineage cells. E2A-deficient mice show a developmental block at the pre-pro-B cell stage (Bain et al., 1994), and fail to rearrange D_H-to-J_H segments due to the absence of *Rag1* expression (Bain et al., 1994; Borghesi et al., 2005). Gene expression studies of E2A-deficient progenitors showed that they do express IL-7Rα, Igβ, and *Igμ*^{0}; EBF is expressed at low levels, and there is no Pax5, CD19, Igα or λ5 expression (Bain et al., 1994; Bain et al., 1997). In addition, the EBF promoter contains an E2A binding site, and E2A to activate the EBF promoter (Roessler et al., 2007; Smith et al., 2002). Moreover, enforced EBF expression in E2A-deficient HSC restores B cell development *in vitro* (Bain et al., 1994; (Seet et al., 2004), although these EBF-expressing pro-B cells cannot proliferate, since E2A is necessary for IL-7R-induced N-Myc upregulation (Seet et al., 2004). All these data suggest that E2A acts upstream of EBF and Pax5 in the transcriptional control of B cell development.

Quite recently, the role of E2A has been studied throughout B cell development. By use of a conditional E2A knockout (KO) mouse bred with different stage-specific Cre lines, E2A was demonstrated to be necessary

for the generation and development of pro-B, pre-B, and immature B cells in the BM and for the development of germinal centre B cells in the spleen, but dispensable for the generation of mature B cells and plasma cells in peripheral organs (Kwon et al., 2008). Gene expression analysis in E2A-deficient pro-B cells showed that *Ebf1*, *Pax5*, *Cd19, Igα, Igβ, Igll1* levels were downregulated compared to those of WT pro-B cells. E2A is therefore also required for maintenance of the gene expression program in committed pro-B cells.

1.4.4. EBF

Early B cell factor (EBF or EBF1) is a member of the COE (Collier-Olf-EBF) family of transcription factors that bind DNA through a zinc-coordination motif found in the N-terminal domain (Hagman and Lukin, 2005). EBF also has a helix-loop-helix domain and a C-terminal activation domain that is not required for protein function (Hagman and Lukin, 2005). EBF is considered the key player in the B cell specification program, although its exact function remains unclear. It is expressed in the haematopoietic system from the CLP stage to mature cells, and its expression is turned off in plasma cells (Hagman and Lukin, 2005; Igarashi et al., 2002). It is also expressed in brain, adipose tissue and olfactory neurons (Hagman et al., 1993; Wang and Reed, 1993). Mice deficient in EBF show a phenotype similar to that of E2A-deficient mice; they do not express many B cell-specific genes such as *Cd79a, Cd79b, Igl11,* and *VpreB*, they show the same pre-pro-B cell stage developmental block, and have no IgH recombination in the BM.

Several functional studies have demonstrated a role for EBF in the B cell specification program. Retroviral expression of EBF in HSC skewed differentiation toward the B cell lineage (Zhang et al., 2003). Ectopic EBF expression rescues B cell development in E2A-deficient HSC (Bain et al., 1994; Seet et al., 2004), in IL-7R-deficient pre-pro-B cells and CLP (Dias et al., 2005; Kikuchi et al., 2005), and in PU1$^{-/-}$ HSC (Medina et al., 2004). In all these cases, Pax5 expression did not rescue B lymphopoiesis, thus indicating that EBF might have additional activities during B cell development in addition to activation of Pax5 expression.

EBF and E2A expression are necessary for the initiation of B cell development; they have been shown to cooperate in the activation of some B cell-specific genes such as *Cd79a, Igl11* and *VpreB* (O'Riordan and Grosschedl, 1999; Sigvardsson, 2000; Sigvardsson et al., 1997). The EBF promoter has an E2A binding site (Smith et al., 2002), indicating that EBF might be a potential E2A target gene. This suggests that EBF and E2A act in a feedback loop important for regulating specificity of B cell fate. Two different EBF promoters were recently identified, EBFα promoter (also known as the distal promoter), which is active in immature B cells, and EBFβ promoter (known as the proximal promoter), active in mature B cells (Roessler et al., 2007). Several transcription factor binding sites have been found on these promoters. The EBFα promoter is regulated by E2A, STAT5 and EBF, whereas the EBFβ promoter is regulated by ETS, PU.1, and Pax5. This study suggests that EBF binding to its promoter acts as an autoregulatory mechanism, whereas Pax5 binding to the EBFβ promoter might amplify the B cell-specific gene expression program and early B cell precursor commitment to the B cell pathway.

A very recent article provided insight into EBF function in the specification of and commitment to the B lineage. Ectopic EBF expression in LMPP and in Pax5-deficient haematopoietic progenitors promotes B cell development, blocks the myeloid potential of LMPP, and restricts alternative lineage potential of Pax5$^{-/-}$ haematopoietic progenitors (Pongubala et al., 2008). This study shows a novel, Pax5-independent function for EBF in the commitment of early precursors to the B cell lineage.

1.4.5. Pax5

Pax5 is one of the nine mammalian Pax transcription factors, but is the only one expressed in the haematopoietic system. The *pax5* gene encodes the B cell-specific activator protein (BSAP or Pax5 protein), a transcription factor that binds to DNA via its N-terminal conserved paired domain (Czerny et al., 1993) and controls gene transcription through a C-terminal module consisting of activating and inhibitory sequences

(Dorfler and Busslinger, 1996). In mouse, it is expressed throughout the B cell lineage from the pro-B stage until its downregulation in plasma cells (Fuxa and Skok, 2007), and in the embryonic midbrain and adult testis (Adams et al., 1992). As shown below, Pax5 has been widely studied and is considered to be the master regulator of commitment of the earliest B cell precursors in the B lineage, and in maintaining B cell identity throughout the life of a B cell before its downregulation in plasma cell differentiation (Fig. I7).

Pax5-deficient mice show a block in B cell development at the transition from pro-B to pre-B cells (Hardy fractions B to C) (Urbanek et al., 1994). Pax5-deficient pro-B cells have been subject of intense study. These cells show normal D_H-to-J_H and $V_{H\ proximal}$-to-DJ_H rearrangements, but impaired $V_{H\,distal}$-to-DJ_H recombination (Nutt et al., 1997). They can also be expanded indefinitely *in vitro* in the presence of IL-7 and a stromal cell line, although they are unable to give rise to mature B cells (Nutt et al., 1997). Unlike WT pro-B cells, Pax5-deficient cells are uncommitted progenitors that, when cultured *in vitro* with the appropriate cytokines, can give rise to functional cells of all other lineages except B lymphocytes (Nutt et al., 1999). Pax5-deficient cells show the same plasticity *in vivo*. When *in vitro*-cultured or *ex vivo* Pax5-deficient cells are transferred into $Rag2^{-/-}$ mice, they provide long-term reconstitution of T cell development (Rolink et al., 1999). Another study showed that when these cells are injected into lethally irradiated mice, they are able to migrate to the BM and reconstitute all haematopoietic lineages except B lymphocytes (Schaniel et al., 2002). Gain-of-function studies have shown that restoration of Pax5 expression in deficient cells suppresses this multi-lineage potential, and cells can differentiate into mature B cells (Nutt et al., 1999), whereas conditional inactivation of Pax5 in pro-B cells reverses lineage commitment and again generates multipotent cells (Mikkola et al., 2002).

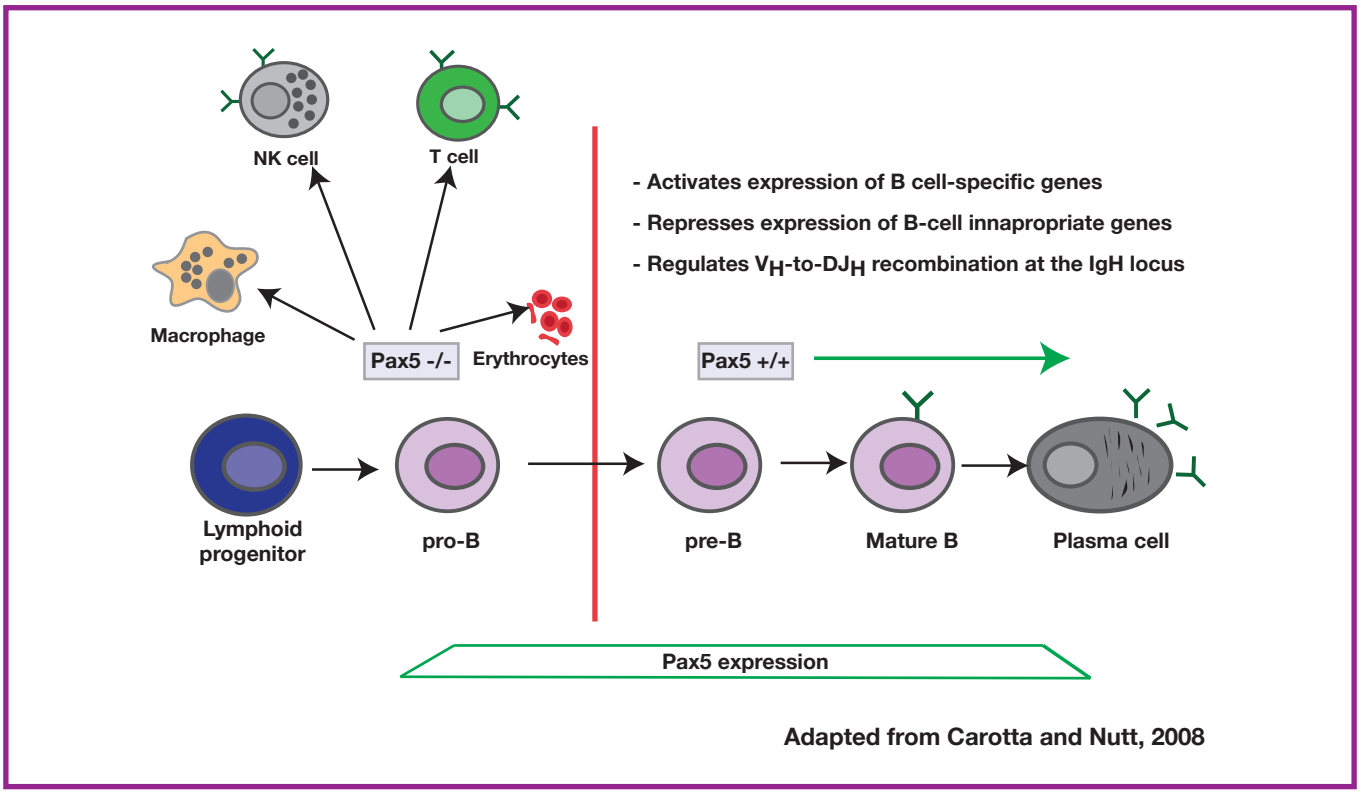

Fig. I7. Biological functions of the Pax5 transcription factor. Pax5 is considered necessary for B lineage commitment. It is expressed throughout B cell development, from pro-B cells until its downregulation in plasma cell differentiation. Pax5-deficiency leads to a block at the pro-B cell stage (vertical red line), these cells are uncommitted and can give rise to cells of other haematopoietic lineages. The biological functions of Pax5 are listed in the right panel.

It was recently shown that when Pax5 is inactivated *in vivo* in mature B cells of peripheral lymphoid organs, cells are able to dedifferentiate to early uncommitted progenitors that can home to BM (Cobaleda et al., 2007). These dedifferentiated progenitors are able to differentiate into macrophages and to rescue T cell development in T cell-deficient mice. Moreover, these B cell-derived T lymphocytes show both IgH and IgL chain rearrangements and can participate as functional T cells in immune reactions (Cobaleda et al., 2007). These dedifferentiated Pax5-deficient cells also develop into aggressive lymphomas with a phenotype similar to Pax5-deficient pro-B cells, but with rearranged IgH and IgL gene segments. Overall, this study provided further evidence of the requirement for continuous Pax5 expression to maintain B cell identity at later differentiation stages.

Pax5 activates the expression of multiple B cell-specific genes, including those encoding the cell surface protein CD19 (Kozmik et al., 1992), the signalling molecule Igα (CD79a) (Fitzsimmons et al., 1996), the surrogate light chains λ5 and VpreB (Okabe et al., 1992), the adaptor protein of pre-BCR signalling BLNK (Schebesta et al., 2002), and the transcription factors N-Myc and LEF-1 (Nutt et al., 1998). Pax5-repressed genes include the macrophage colony stimulating-factor receptor (M-CSFR, encoded by *Csf1R*) (Nutt et al., 1999), Notch1 (Souabni et al., 2002), and *FLt3* (Holmes et al., 2006).

All of these studies suggest that the transcriptional control of early B cell development is not a linear mechanism in which each transcription factor actis at a given developmental stage. It is becoming clear that the commitment of lineage-specific progenitors to a particular fate is dependent on dose, combination, and cross-competition among these factors. Moreover, there is a continuous need for the expression of some of these transcription factors to reinforce the commitment process throughout a B cell's life.

1.5. c-Myc and B lymphopoiesis

The role of c-Myc in B cell development is poorly understood, mainly because of the redundancy of related transcription family members that might compensate the *c-myc* expression defect. Only *c-* and *N-myc* are expressed in mammalian B lymphocytes; in addition, *c-myc* is expressed at different levels throughout the differentiation process. Expression begins at the pro-B cell stage, induced in response to cytokines such as IL-7 (Morrow et al., 1992). It is also expressed, together with *N-myc*, in large pre-B cells during maturation and expansion to small pre-B cells (Zimmerman and Alt, 1990). Thereafter, only *c-myc* is expressed in immature and mature B cells after B cell activation.

Studies of several B cell lines have shown that *c-myc* up- or downregulation results in apoptosis (Thompson, 1998). In immature B cell lines, such as WEHI-231 or CH31, apoptosis is induced after anti-IgM crosslinking. BCR stimulation induces a transient increase in *c-myc* levels, which return to basal levels after 4 hours. In these cell lines, stabilisation of c-Myc levels or c-Myc overexpression protects cell from apoptosis induced by anti-IgM treatment (Fischer et al., 1994; Wu et al., 1996). c-Myc downregulation is thus necessary for immature B cell lines to undergo apoptosis. In mature B cell lines, on the other hand, in which *c-myc* expression is very low, BCR stimulation induces upregulation of c-Myc, eventually leading cells to apoptosis (Kelly et al., 1983).

Gain-of-function studies using a murine model of lymphomagenesis (Eμ-myc mice) have been used to study c-Myc in B cells. Constitutive expression of a *c-myc* transgene under the control of the heavy chain enhancer (Eμ) results in impaired B lymphopoiesis, reflected in an increase in pro- and pre-B (IgM^-) cells and in a reduction of immature and mature B cells (IgM^+) in BM. A reduction in spleen B lymphocytes is also observed, which is attributed to impaired B cell development in the BM or to an increase in apoptotic cells due to inappropriate *c-myc* expression (Iritani and Eisenman, 1999). In addition, B cells from both BM and spleen show an increase in cell size, which is independent of their developmental stage and is found at all cell cycle phases. This cell size increase correlates with increased protein synthesis in normal pre-transformed B lymphocytes at all B cell developmental stages (Iritani and Eisenman, 1999).

The use of mouse conditional KO models has provided greater understanding of the *in vivo* function of the proto-oncogene *c-myc* in B lymphocytes. c-Myc was shown to affect B cell proliferation and apoptosis. Initial studies showed that c-Myc is necessary for proliferation of primary B cells (de Alboran et al., 2001). When c-Myc-deficient mature B lymphocytes are cultured with mitogenic stimuli, cells accumulate in the G0/G1 phase and show a shortened S phase. These cells have increased levels of the cell cycle inhibitor p27, which could explain the proliferation impairment at least in part (de Alboran et al., 2001). c-Myc-deficient B lymphocytes are also more resistant to various apoptotic stimuli. Non-activated c-Myc-deficient B cells are resistant to spontaneous cell death, whereas activated cells are resistant to CD95-induced cell death (de Alboran et al., 2003). When c-Myc-deficient B cells are activated by mitogenic stimuli, they express normal surface levels of activation markers, but reduced CD95 and CD95L levels. The role of c-Myc in B cell development has recently been studied using double *c-* and *N-myc* KO mice, showing that conditional *myc* deletion notably inhibits B cell development at the pro-B to pre-B cell transition (Habib et al., 2007). Another approach showed that c-Myc expression rescues the developmental block in differentiation in the absence of pre-BCR formation. As Ca^{2+} signalling is an important pre-BCR-induced event, these authors also analyzed whether c-Myc expression had an effect on Ca^{2+} mobilisation; they found that Myc might promote proliferation and differentiation of primary B cells by amplifying Ca^{2+} signalling, which enables expression of both Myc and Ca^{2+}-regulated genes, which are essential for cell division and differentiation.

Aims

2. Aims

1. To characterise the developmental block in B cell differentiation due to *c-myc* inactivation.

2. To identify potential c-Myc target genes in B lymphocyte differentiation.

3. To perform gain-of-function studies with potential c-Myc targets to rescue the phenotype in c-Myc-deficient B lymphocytes.

Materials & Methods

3. Materials & Methods

3.1. Mice.

Generation of c-myc$^{fl/fl}$; mx-cre^{+} mice was described previously (Baena et al., 2005). To generate c-myc$^{fl/fl}$; mb1$^{cre/+}$, we bred c-myc$^{fl/fl}$ with mb1$^{cre/+}$mice (Hobeika et al., 2006) and crossbred their progeny to yield homozygous (c-myc$^{fl/fl}$; mb1$^{cre/+}$) and control mice (c-myc$^{fl/+}$; mb1$^{cre/+}$ or c-myc$^{fl/fl}$; mb1$^{+/+}$). Briefly, c-myc$^{fl/fl}$; mb1$^{cre/+}$ were bred with rosa26$^{gfp/gfp}$ mice (Mao et al., 2001), and progeny crossbred to yield homozygous c-myc$^{fl/fl}$; mb1$^{cre/+}$; rosa26$^{gfp/gfp}$ or heterozygous c-myc$^{fl/+}$; mb1$^{cre/+}$; rosa26$^{gfp/gfp}$ mice.

Briefly, c-myc$^{fl/fl}$; mx-cre^{+} or c-myc$^{fl/fl}$; mb1$^{cre/+}$ mice were bred with Ik$^{neo/+}$ mice (Souabni et al., 2002) to generate Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+} or Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb1$^{cre/+}$, respectively. Progeny was crossbred to generate homozygous (Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+} or Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb1$^{cre/+}$) control mice (Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{-} or Ik$^{neo/+}$; c-myc$^{fl/+}$; mb1$^{cre/+}$).

Mice were genotyped using a PCR-based analysis of tail genomic DNA (de Alboran et al., 2004; Baena et al., 2005).

3.2. Genomic PCR.

Primers used to amplify the floxed allele (530 bp product) were FloxS (5'- GCC CCT GAA TTG CTA GGA AGA CTG-3') and FloxA (5'-CCG ACC GGG TCC GAG TCC CTA TT-3'). The deleted allele (Δ, 600 bp) was amplified with primers Null S (5'-TCG CGC CCC TGA ATT GCT AGG AA-3') and Null A (5'-TGC CCA GAT AGG GAG CTG TGA TAC TT-3'). The mx-cre transgene (269 bp product) was amplified with primers SF-4 (5'- GCA TAA CCA GTG AAA CAG CAT TGC TG-3') and 69-R (5'-GGA CAT GTT CAG GGA TCG CCA GGC G-3').

Primers hcre-DIR (5'- ACC TCT GAT GAA GTC AGG AAG AAC-3'), hcre-REV (5'- GGA GAT GTC CTT CAC TCT GAT TCT-3'), mb1in1 (5'- CTG CGG GTA GAA GGG GGT C-3') and mb1in2 (5'- CCT TGC GAG GTC AGG GAG CC-3') were used to amplify mb1-cre (hcre-DIR and hcre-REV, 600 bp PCR product) and mb1-WT alleles (mb1in1 and mb1in2, 550 bp PCR product). The knock-in allele (Ik$^{neo/+}$) was identified by a 780 bp PCR fragment using Ik^{neo}-5' (5'- GCG GAG CTC CTC AGG TGC AGG CTG CCT ATC- 3') and Ik^{neo}-3' (5'- CGC GTC GAC GCT AAC ATC CTG AGG GAC TGT-3') primers.

3.3. PipC injections.

To induce c-myc deletion in c-myc$^{fl/fl}$; mx-cre^{+} and Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+} mice, 4- to 6-week-old mice were given three intraperitoneal (i.p.) injections of polyinosinic-polycytidylic acid (pIpC, Amersham) (150 μg each, or 250 μg each for in vitro differentiation experiments) at two-day intervals and analyzed 3 or 5 days after the last dose.

3.4. Flow cytometry sorting and analysis.

Single-cell suspensions of bone marrow (BM) cells, isolated from femurs and tibias, and of spleens were resuspended in PBS containing 2% FBS. Cells were first incubated for 10 min on ice with FcBlock (Beckman) to avoid non-specific antibody binding. Subsequently, cells were incubated (15 min, on ice) with primary antibodies, washed, and biotin-stained cells were incubated with secondary antibodies (15 min, on ice). Primary antibodies were B220 conjugated to PeCy7 (Bioscience), FITC or APC (Becton Coulter), IgM-PE or -biotin (Southern Biotechnologies), CD19-APC (Becton Coulter), and

CD25-PE, CD43-biotin, CD117-PE or pre-BCR-biotin (all from Pharmingen). Streptavidin-APC (Pharmingen) or -ECD (Immunotech) were used as secondary antibodies. B lymphocytes from BM were sorted as $B220^{+}IgM^{-}$ (pro- and pre-B cells), $B220^{lo}IgM^{+}$ (immature cells) and $B220^{hi}IgM^{+}$ (mature cells) on a FACS Coulter flow cytometer. The purity of the sorted populations was verified by flow cytometry reanalysis as >97%.

3.5. Apoptosis analysis.

BM cells were isolated and stained as described above, and apoptosis analysis was performed using FITC- or PE-annexin V (Immunotech and Becton Coulter, respectively) and propidium iodide (PI) staining according to the manufacturer's protocol.

3.6. BrdU labelling.

Analysis of BrdU incorporation into BM cell populations was assessed 2 h after a single intravenous BrdU injection (1 mg/15 g body weight, Sigma). $B220^{+}IgM^{-}$ (pro-B cells), $B220^{lo}IgM^{+}$ (immature cells) and $B220^{hi}IgM^{+}$ (mature cells) cells were sorted from BM, and BrdU incorporation measured using FITC- or PE- anti-BrdU monoclonal antibody (Becton Dickinson, Mountain View, CA, USA) and PI, following standard protocols.

3.7. Gene expression analysis.

RNA from sorted $B220^{+}IgM^{-}$ cells (1.5-2 x 10^5 cells) was extracted using the RNeasy Micro kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was primed with random primers (Promega) from whole sample RNA using the Reverse Transcription System (Promega). For real-time PCR analysis, 2.5 µl cDNA was mixed with primers and SYBR Green PCR master mix (Becton Dickinson) following the manufacturer's instructions. cDNA was used in a tenfold dilution series (10- and 100-fold). Reverse transcription-PCR was on an ABI Prism 7700HT and was analyzed with SDS 1.9 sequence detection system (Applied Biosystems). All oligonucleotides were designed to yield 50-100 bp PCR fragments. Oligonucleotides for c-myc were c-mycRT-5' (5'- TGC CTC TTC TCC ACA GAC AC-3') and c-mycRT-3' (5'- TCT GTA CCT CGT CCG ATT C-3'), for Tcfe2a, E2A-F (5'- AAG AGG ACA AGA AGG ACC TGA A-3') and E2A-R (5'- TTA TTG GCC ATA CGC CTC TC-3'), EBF1-F (5'- CAG GAA ACC CAC GTG ACA T-3') and EBF-R (5'- CCA CGT TGA CTG TGG TAG ACA-3') for ebf; primers for cd19 were CD19-F (5'- AAG GTC ATT GCA AGG TCA GC-3') and CD19-R (5'- CTG GGA CTA TCC ATC CAC CA) ,for actin primers were actin1 (5'- AAG GAG ATT ACT GCT CTG GCT CCT A-3') and actin2 (5'- ACT CAT CGT ACT CCT GCT TGC TGA T-3'), and 28s-5' (5'- TGC CAT GGT AAT CCT GCT CA-3') and 28s-3' 85'- CCT CAG CCA AGC ACA TAC ACC-3') for 28s.

Pre-designed Applied Biosystems Micro Fluidic cards containing cDNA samples and reagents were run on an ABI Prism 7900HT. Data were analyzed with SDS2.2 sequence detection systems.

3.8. Cell isolation and culture.

B cell cultures were established from BM cell suspensions from 4- to 8-week-old mice by depletion using antibodies to IgM (Southern Biotechnologies) and Streptavidin Dynabeads (Invitrogen). Purity of cells after magnetic separation was confirmed by flow cytometry as >98%. Cells were cultured in 24-well plates (2 x 10^6 cells/ml) in Iscove's Modified Dulbecco's Medium (IMDM) containing 2% heat-inactivated foetal bovine serum (FBS), 1 mM L-glutamine, 1 mM penicillin/streptomycin, 0.03% w/v primatone RL (Sigma), 50 µM □-mercaptoethanol) and supplemented with recombinant murine stem cell factor (SCF, 10 ng/ml), recombinant murine FLt3 ligand (FLt3L, 10 ng/ml) and recombinant murine IL-7 (10 ng/ml), all from PeproTech.

To isolate lineage-negative precursors, BM cells were incubated with a cocktail of biotinylated antibodies to lineage markers (B220, IgM, CD4, CD8, Ter119, Gr1 and CD11b, all from Pharmingen) and Streptavidin Dynabeads (Invitrogen). Cells were cultured at 10^6 cells/ml in 24-well plates in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated FBS, 1 mM

L-glutamine and supplemented with recombinant murine stem cell factor (SCF, 5 ng/ml), recombinant murine IL-6 (2.5 ng/ml) and murine leukemia inhibitory factor (LIF, 10^3 units/ml).

Cells were incubated in standard conditions (37°C, 5% CO_2, 96% relative humidity) for up to one week and the percentage of pro-B, immature and mature B lymphocytes was assessed by FACS at days 4 and 7 of culture.

3.9. Retrovirus production and transduction.

PLAT-E cells were maintained in DMEM containing 10% heat-inactivated FBS, 1 mM L-glutamine, 1 mM penicillin/streptomycin, 1 µg/ml puromycin (Clontech) and 10 µg/ml blasticidin (Invitrogen). PLAT-E cells were seeded (2 x 10^6 cells) in 6-cm plates, 18-24 h before transfection with FuGene 6 reagent (Roche Diagnostics). MIG-RI or MIG-EBF (3 µg) were transfected to each plate containing PLAT-E cells. After 24 h, medium was replaced by complete IMDM without cytokines. Retroviral supernatants were collected 48 h post-transfection and filtered through a 45 µm low protein-binding- syringe filter (Pall, Life Sciences).

Pre-stimulated BM cells and lineage precursors were transduced by spin infection after 1 day in culture. Cells were resuspended in 1 ml of fresh retroviral supernatant, supplemented with 10 µg/ml polybrene (Sigma-Aldrich) and cytokines at the same concentration as above. Cells were centrifuged at 2000 rpm (90 min, 32ºC). After centrifugation, cells were placed at the incubator (3-4 h), after which medium was replaced by fresh IMDM plus cytokines (SCF, Flt3L and IL-7, 10 ng/ml each). Transduction efficiency was monitored by FACS at 48 to 72 h post-infection.

3.10. Titration of retroviral supernatants.

Retroviral supernatants generated on 293T cells were titrated in NIH3T3 fibroblasts. At 24 h before infection 1.5 x 10^5 cells were plated in each well of a 6-well plate. On the day of infection, viral supernatants were diluted 10-fold in complete DMEM medium supplemented with polybrene (10 µg/ml, Sigma-Aldrich) and added to target cells. Cells were incubated (6 to 8 h) and medium was replaced by fresh DMEM. At 48 h post-infection, cells were collected and analyzed by flow cytometry for GFP expression.

The following formula was applied to calculate virus titre: titre (IU/ml) = number of target cells at day of infection X (percentage of GFP-positive cells)/100 X dilution factor.

3.11. Clonogenic assays.

Purified $B220^+CD43^+$ cells from each genotype (5 x 10^3 cells/plate) were seeded into methylcellulose-containing media. To assess generation of BFU-E, cells were plated into MethoCult 3234 (Stem Cell Technologies, Vancouver, Canada) supplemented with 10 ng/ml murine IL-3 (Biosource, Worcester, MA), 3 U/ml human recombinant erythropoietin (Stem Cell Technologies) and 50 ng/ml murine SCF (Peprotech). For generation of CFU-GM and CFU-preB precursors, cells were seeded into MethoCult M3534 and MethoCult M3630, respectively (both from Stem Cell Technologies). CFU-GM and CFU-preB colonies were scored on day 7, and BFU-E colonies on day 9.

Results

4. Results

4.1. Animal models for the study of *c-myc* function in B lymphocytes

Germ line inactivation of *c-myc* causes embryonic lethality at day E9.5-10.5. For this reason, the development of conditional knockout mice enables study of the function of this proto-oncogene *in vivo*. We used the *c-myc*$^{fl/fl}$ mice (de Alboran et al., 2001), based on the cre-loxP strategy; breeding them with mice carrying a stage-specific *Cre* recombinase gene permits analysis of the *in vivo* function of *c-myc* in early B lymphopoiesis.

To study the role of the proto-oncogene *c-myc* in B cell development, we generated two animal models that delete *c-myc* in developing B lymphocytes, in either a constitutive or an inducible way. Generation of *c-myc*$^{fl/fl}$ mice was previously described (de Alboran et al., 2001). Briefly, *loxP* sites were inserted between exons 2 and 3 in the mouse germ line *c-myc* locus (Fig. R1). Mice homozygous for this mutation showed no phenotype in the absence of the *Cre* recombinase (Baena et al., 2005). Activation of *Cre* recombinase leads to complete inactivation of the *c-myc* gene by deletion of the sequence flanked by the two loxP sites (de Alboran et al., 2001)(Fig. R1).

4.1.1. Constitutive model: *c-myc*$^{fl/fl}$; *mb-1*$^{cre/+}$

To study *c-myc* function in B lymphocytes, we bred *c-myc*$^{fl/fl}$ with the *mb-1*$^{cre/+}$ knock-in mice (Hobeika et al., 2006), which have the *Cre* recombinase gene under the control of the *mb-1* promoter. The *mb-1* gene encodes the Igα subunit of the pre-B cell receptor (pre-

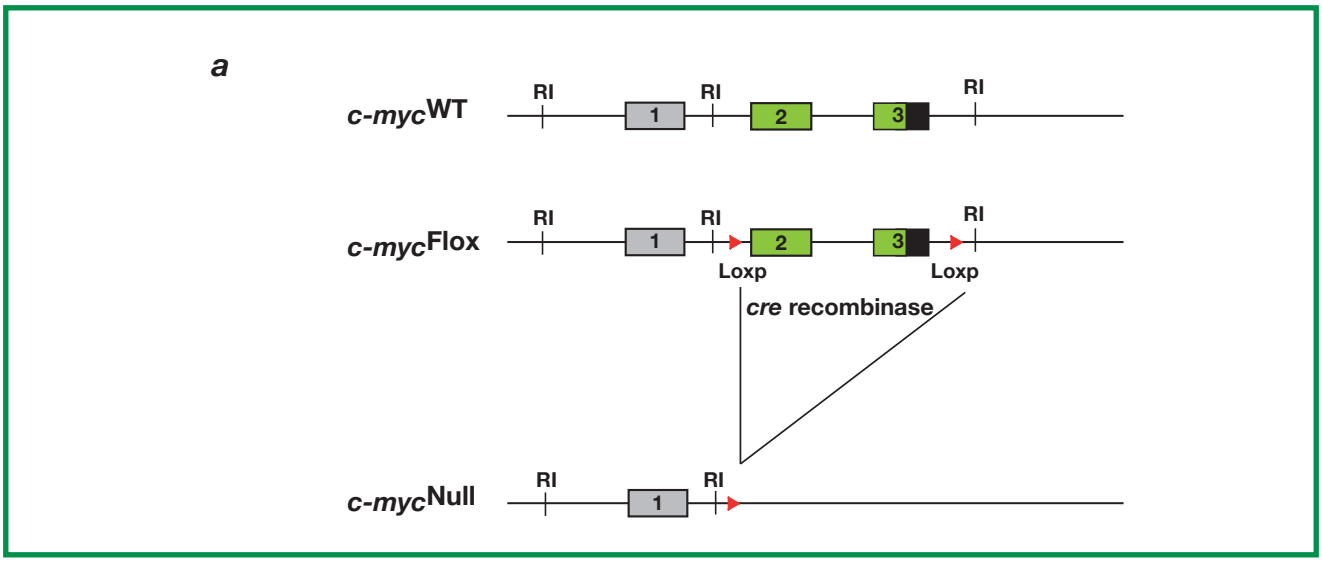

Fig. R1. Structure of the targeted *flox* locus. The *c-myc* gene flanked by two *lox*P sites inserted between exons 2 and 3. *Cre*- mediated recombination generated a null *c-myc* allele that lacks exons 2 and 3. RI, EcoRI restriction sites; red arrowheads indicate loxP sites; green rectangles show coding exons; black rectangles show non-coding exons.

BCR) and is expressed exclusively in B cells beginning at the early pro-B cell stage in the BM (Hobeika et al., 2006). F2 generation intercrosses generated homozygous *c-myc*$^{fl/fl}$; *mb-1*$^{cre/+}$ and littermate controls *c-myc*$^{fl/+}$; *mb-1*$^{cre/+}$ (Fig.R2a). *c-myc*$^{fl/fl}$; *mb-1*$^{cre/+}$ mice inactivate *c-myc* constitutively and specifically in B lymphocytes, making them a perfect mouse model in which to study the role of the proto-oncogene *c-myc* in B cell development.

To track c-Myc-deficient B cells by the expression of green fluorescent protein (GFP), *c-myc*$^{fl/fl}$; *mb-1*$^{cre/+}$ mice were bred with the *rosa26*$^{gfp/gfp}$ reporter mice (Mao et al., 2001)(Fig. R2a), which express

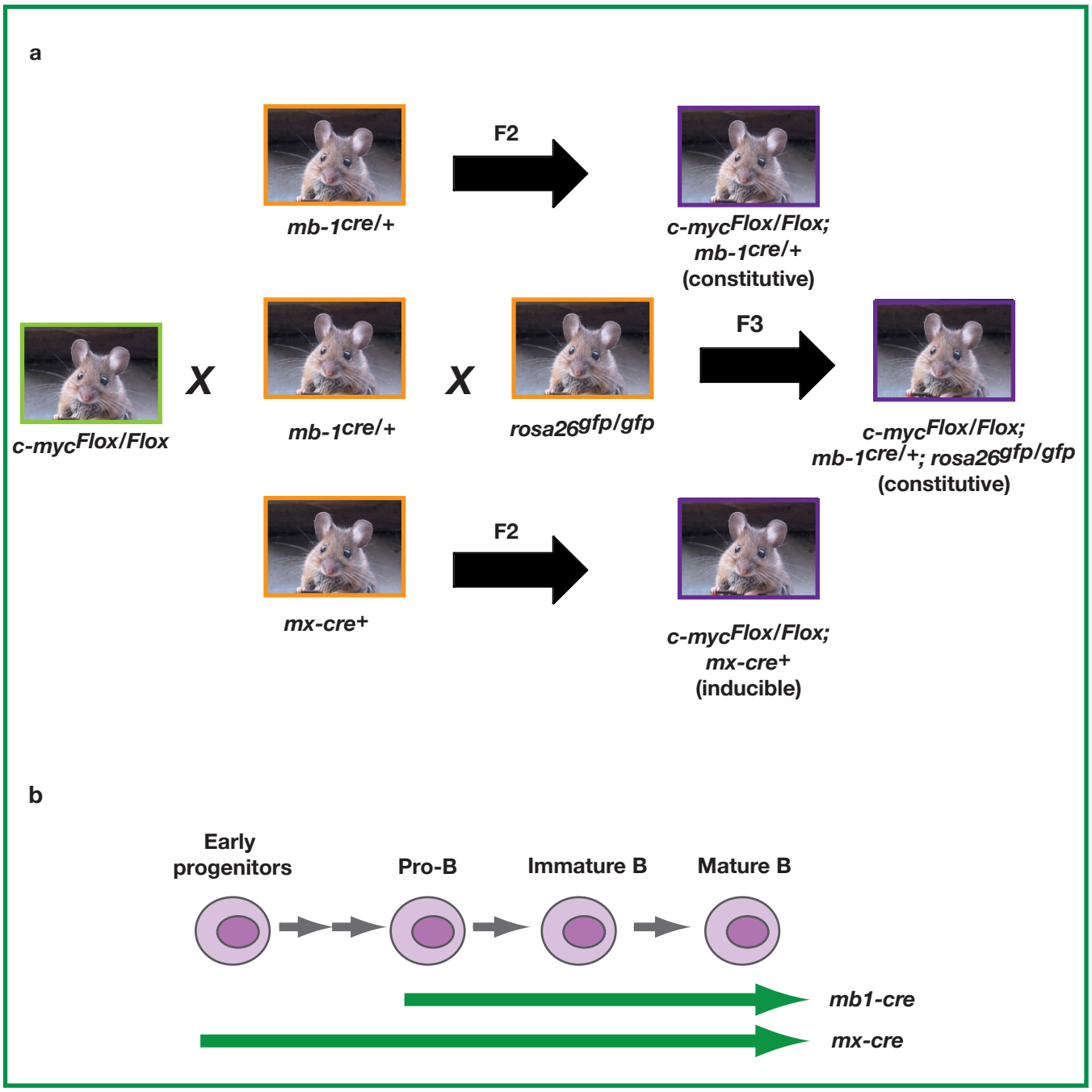

Fig. R2. Scheme showing the mouse models used to study *c-myc* function in B cell development. (a) *c-myc*$^{flox/flox}$ mice were bred with different *Cre* recombinase strains to produce several mouse models that inactivate *c-myc* at different time points during B cell development. (b) Time-course *c-myc* inactivation in BM in two of the mouse models.

enhanced GFP (eGFP) under the endogenous promoter of the *rosa26* gene. *Cre* recombinase expression by the endogenous *mb-1* locus leads to deletion of both the *c-myc* locus and the stop codon inserted in the *egfp* gene in immature B lymphocytes. This strategy allows us to distinguish c-Myc-deficient cells from undeleted cells based on GFP expression, and therefore to select and isolate c-Myc-deficient B cells (de Alboran et al., 2004).

4.1.2. Inducible model: *c-myc$^{fl/fl}$; mx-cre^{+}* mice

c-myc$^{fl/fl}$; mx-cre^{+} mice have been described (Baena et al., 2005). Briefly, *c-myc$^{fl/fl}$* mice were bred with the *mx-cre* transgenic mice (Kuhn et al., 1995), which carry the *Cre* recombinase gene under the control of the inducible promoter of the murine *mx*1 gene. Mx1 is part of the defence against viral infections and is silent in healthy mice. The *mx*1 promoter is activated transiently in several tissues after injection of α or β interferon or polyinosinic-polycytidylic acid (pIpC), a synthetic double-stranded RNA that acts as an interferon inducer. The F2 generation originated homozygous *c-myc$^{fl/fl}$; mx-cre^{+}* mice, which inactivate the *c-myc* gene following pIpC treatment (Fig. R2a). After pIpC injection, *c-myc$^{fl/fl}$; mx-cre^{+}* mice show *c-myc* inactivation in several organs. Efficient deletion of *c--myc* (80-100%) is found in bone marrow (BM) (Baena et al., 2005), making this animal model suitable for the study of *c-myc* in BM hematopoiesis, and therefore of early B lymphopoiesis.

It is important to note the differences between these two models. Whereas *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice begin to inactivate *c-myc* specifically at the pro-B cell stage, *c-myc$^{fl/fl}$; mx-cre^{+}* delete the *c-myc* gene from the earliest progenitors in the BM (Fig. R2b).

4.2. c-Myc function in B cell development

The *c-myc* proto-oncogene is expressed in both immature and mature B cells (Leider and Melamed, 2003). Our first objective was therefore to characterise the different B cell subsets found in c-Myc-deficient BM. B lymphopoiesis in the BM can be followed by the expression of several cell surface markers, as well as by the immunoglobulin gene rearrangement that takes place in a sequential and ordered way. Among the B220^{+}IgM^{-}CD43^{+} population, several subsets are identified: Fraction A (Hardy et al., 1991) or pre-pro-B cells, the least-committed B cell population, pro-B cells (or Fraction B/C), and pre-B cells (Fraction D). Within the B220^{+}IgM^{+}CD43^{+} population, immature B (Fraction E) and mature B cells (Fraction F) are found.

4.2.1. c-Myc is necessary for B cell development

To study *c-myc* function in B cell development, we analysed 5- to 10-week-old *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice that, as explained above, delete *c-myc* specifically in B cells at the pro-B cell stage. To characterise the distinct B cell subsets, we studied the surface expression markers defined by Hardy and colleagues (Hardy et al., 1991). Analysis of total BM (tBM) showed that both the B220^{+}CD43^{-} and B220^{+}IgM^{+} populations were absent in the BM of *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice (Fig. R3a). In wild-type mice, a B220 versus IgM plot (Fig. R3a, upper panel) indicates three populations: the B220^{+}IgM^{-} population comprised of Hardy Fractions A-D (or pro- and pre-B cells), B220^{low}IgM^{+}, corresponding to Fraction E (or immature B cells), and B220^{hi}IgM^{+} Fraction F (or mature B cells). In *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice, this plot showed that c-Myc-deficient B lymphocytes were unable to differentiate beyond the B220^{+}IgM^{-} stage of B cell development. Absolute numbers showed that the B220^{+} population was reduced by three-fold (4.5 x 10^{6} vs. 1.3 x 10^{6} cells), whereas B220^{+}IgM^{-} cells were reduced by two-fold (2.4 x 10^{6} vs. 1.2 x 10^{6} cells) in the BM of *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice compared to control littermates (Fig. R3b). The B220 versus CD43 plot (Fig. R3a, lower panel) permits a more detailed analysis, as it differentiates Fractions A, B, C and C' (found in the B220^{+}CD43^{+} population) from Fractions D and E (in the B220^{+}CD43^{+} population). These data showed that c-Myc-deficient B lymphocytes were unable to differentiate beyond the Fraction C stage.

Genomic PCR on sorted B220^{+}IgM^{-}GFP^{+} cells

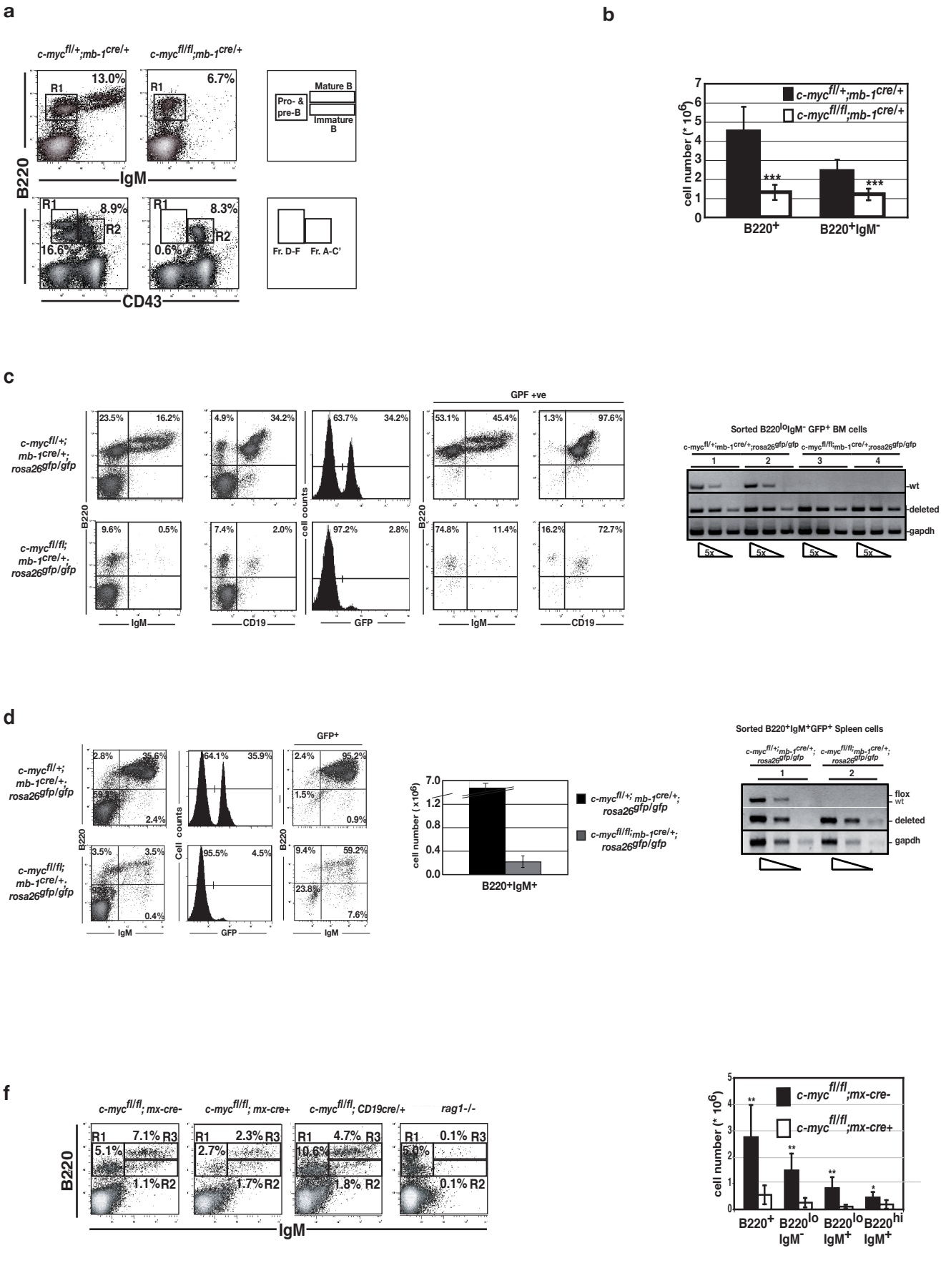
a
c-myc fl/+;mb-1 cre/+
c-myc fl/fl;mb-1 cre/+
R1
13.0%
6.7%
B220
IgM
Mature B
Pro- & pre-B
Immature B
R1
8.9%
R2
16.6%
8.3%
0.6%
CD43
Fr. D-F
Fr. A-C'
b
cell number (* 10^6)
c-myc fl/+;mb-1 cre/+
c-myc fl/fl;mb-1 cre/+
***
B220+
B220+IgM-
c
GPF +ve
c-myc fl/+; mb-1 cre/+, rosa26 gfp/gfp
c-myc fl/fl; mb-1 cre/+, rosa26 gfp/gfp
23.5%
16.2%
4.9%
34.2%
63.7%
34.2%
53.1%
45.4%
1.3%
97.6%
9.6%
0.5%
7.4%
2.0%
97.2%
2.8%
74.8%
11.4%
16.2%
72.7%
B220
cell counts
IgM
CD19
GFP
Sorted B220loIgM- GFP+ BM cells
c-myc fl/+;mb-1 cre/+;rosa26 gfp/gfp
c-myc fl/fl;mb-1 cre/+;rosa26 gfp/gfp
1
2
3
4
wt
deleted
gapdh
5x
d
GFP+
2.8%
35.6%
64.1%
35.9%
2.4%
95.2%
1.5%
0.9%
3.5%
3.5%
95.5%
4.5%
9.4%
59.2%
23.8%
7.6%
0.4%
Cell counts
IgM
GFP
cell number (x10^6)
B220+IgM+
c-myc fl/+; mb-1 cre/+; rosa26 gfp/gfp
c-myc fl/fl;mb-1 cre/+; rosa26 gfp/gfp
Sorted B220+IgM+GFP+ Spleen cells
flox
wt
deleted
gapdh
f
c-myc fl/fl; mx-cre-
c-myc fl/fl; mx-cre+
c-myc fl/fl; CD19cre/+
rag1-/-
R1
7.1% R3
5.1%
1.1%R2
2.3% R3
2.7%
1.7%R2
4.7% R3
10.6%
1.8% R2
0.1% R3
5.0%
0.1% R2
B220
IgM
cell number (* 10^6)
c-myc fl/fl;mx-cre-
c-myc fl/fl;mx-cre+
B220+
B220lo IgM-
B220lo IgM+
B220hi IgM+

Fig. R3. c-Myc is required for B cell development. (a) Total BM suspensions of *c-myc*$^{fl/fl}$*; mb--1*$^{cre/+}$ mice and control littermates were stained with antibodies to B220, IgM and CD43 to characterise immature B cell populations by flow cytometry. (b) Absolute numbers of B lymphocytes in BM of *c-myc*$^{fl/+}$*; mb-1*$^{cre/+}$(black bars; n = 8) and *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ (open bars; n = 8). P-value *** p<0.001. (c) Representative BM staining with antibodies to B220, IgM and CD19 to study B lymphocytes in homozygous *c-myc*$^{fl/fl}$*;-mb-1*$^{cre/+}$*; rosa26*$^{gfp/gfp}$ mice and control littermates. GFP-positive cells were gated and analysed for surface expression of B220, IgM and CD19 to define the different cell subsets: B220^{+}IgM^{-} (pro- and pre-B cells), B220^{lo}IgM^{+} (immature B cells), B220^{hi}IgM^{+} (mature B cells), and B220^{+}CD19^{+} (pro-B cells). (d) (left) Staining of spleen cells from *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$*; rosa26*$^{gfp/gfp}$ (black bars) and littermate controls (grey bars). GFP-positive cells were gated and studied for the surface expression of B220 and IgM, to study B220^{+}IgM^{+} cells in the spleens of these mice. (right) Absolute numbers of spleen B220^{+}IgM^{+} cells from *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$*; rosa26*$^{gfp/gfp}$ (n=5) and control littermates (n=5) (e) Genomic PCR of sorted B220^{+}IgM^{-}GFP^{+} BM cells and B220^{+}IgM^{+}GFP^{+} cells from *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$*; rosa26*$^{gfp/gfp}$ mice and control littermates. (f) (left) Representative staining of BM cells from pIpC-treated *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice and control littermates. (right) Absolute numbers of B lymphocytes in BM of *c-myc*$^{fl/+}$*; mx-cre*$^{+}$ (black bars; n = 8) and *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice (open bars; n = 8). All data are representative of at least three independent experiments.

from *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$*; rosa26*$^{gfp/gfp}$ mice showed that *c-myc* was deleted in these cells (Fig. R3c). FACS analysis of various cell surface markers indicated that GFP-positive cells in these mice were exclusively B cells (Fig. R3c), indicating that *c-myc* is deleted solely in B cells.

We next characterised B cells in the spleen of *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$*; rosa26*$^{gfp/gfp}$ mice. For this purpose, splenocytes were stained with fluorescence-labelled antibodies to B220 and IgM surface markers, which allow the characterisation of B lymphocytes in the spleen. Staining showed that spleen mature B cells (B220^{+}IgM^{+}) were greatly reduced in homozygous mice compared to control littermates (3.5% vs. 35.6%; 10.2-fold decrease) (Fig. R3d). Genomic PCR on sorted B220^{+}IgM^{-}GFP^{+} cells showed that that *c-myc* was deleted in these cells (Fig. R3e). There are two possible explanations for this phenomenon. One is that some cells that inactivate *c-myc* are able to differentiate, migrate into the blood stream and colonise the spleen; the other is that since the *mb-1* gene is expressed at every stage of B cell development and activation, excluding terminally differentiated plasma cells (Hobeika et al., 2006), B220^{+}IgM^{+} cells in the homozygous mouse spleen could escape *c-myc* deletion at the pro-B cell stage and inactivate *c-myc* once they reach the spleen. Concurring with the staining results, the absolute numbers of B220^{+}IgM^{+} cells showed a 16.8-fold decrease in *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$*; rosa26*$^{gfp/gfp}$ compared to control *c-myc*$^{fl+l}$*; mb-1*$^{cre/+}$*; rosa26*$^{gfp/gfp}$ mice (Fig. R3d).

B lymphocytes can be maintained and expanded *in vitro* when they are co-cultured with a stromal cell line and certain exogenous cytokines. Flt3 ligand, stem cell factor (SCF), and IL-7 are important cytokines for B cell development (Mackarehtschian et al., 1995) (Sitnicka et al., 2003).

To determine whether the B220^{+}IgM^{+} cells in the spleens of c-Myc-deficient mice were generated in the absence of *c-myc*, BM cells were depleted of IgM-positive cells and cultured with the Flt3L/SCF/IL-7 cytokine cocktail. After 3 or 4 days in culture, *in vitro* differentiation was assessed by FACS analysis. We performed these experiments with both *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ and *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$*; rosa26*$^{gfp/gfp}$ BM cells. In both cases, we found that after 3 days in culture, there was substantial B cell enrichment in the cultures, as assessed by B220 vs. IgM and B220 vs. CD19 plots. Whereas in the case of WT cells, differentiation proceeded through B220^{+}IgM^{+} and B220^{+}CD19^{+} cells, in c-Myc-deficient BM cells were blocked at the B220^{+}IgM^{-} and B220^{+}CD19^{-} developmental stage (Fig. R4a, b).

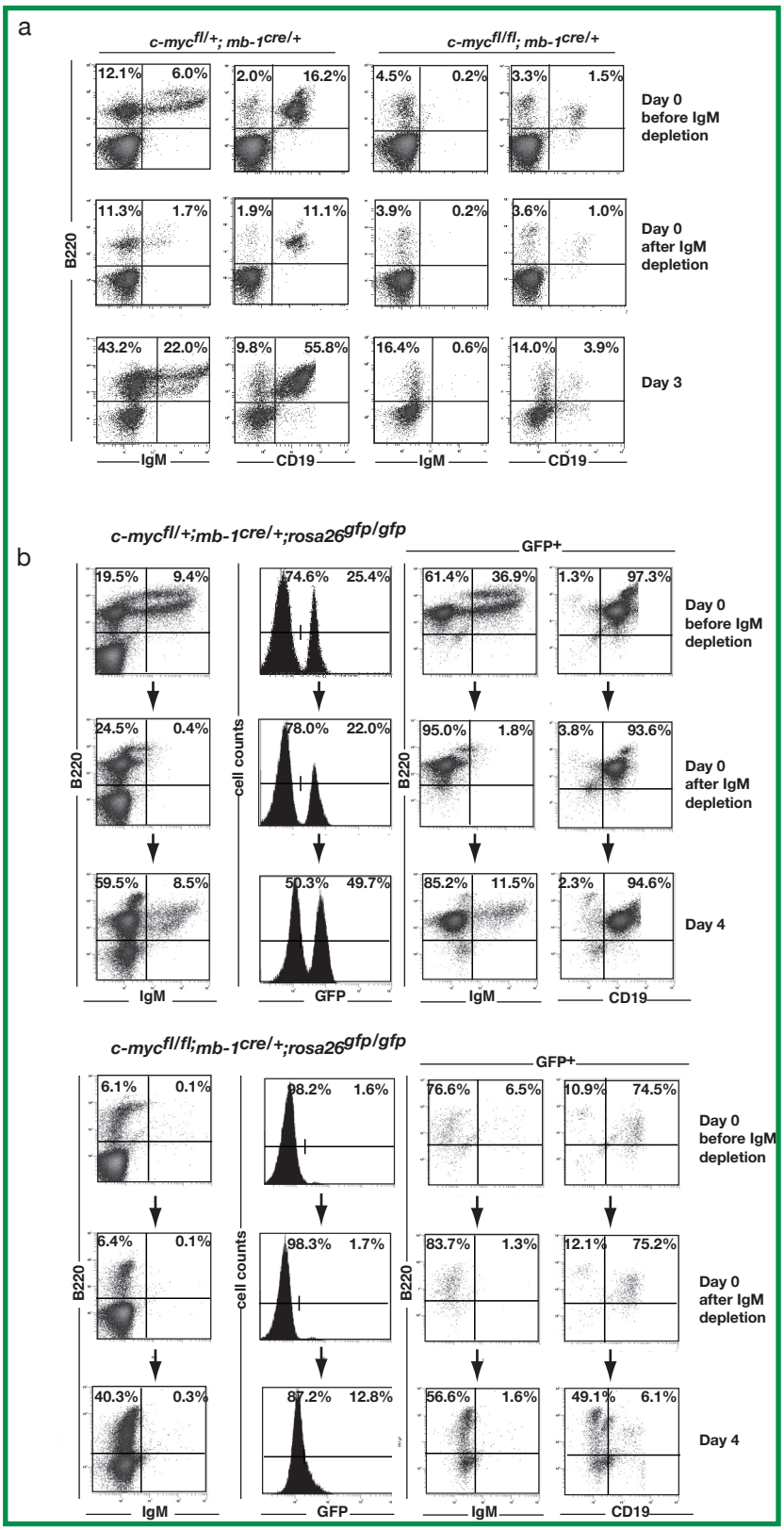

Fig. R4. Differentiation of c-Myc-deficient cells *in vitro*. (a) IgM$^+$ cells from BM of *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice and control littermates were depleted using magnetic beads. Cells were cultured in IMDM medium supplemented with SCF, Flt3L and IL-7 and allowed to differentiate for three days. After this period, cells were stained with antibodies to B220, IgM and CD19, and differentiation was assessed by flow cytometry analysis. (b) As in (a), using *c-myc$^{fl/fl}$; mb-1$^{cre/+}$; rosa26$^{gfp/gfp}$* mice and age-matched controls.

These results indicate that c-Myc is required for B cell differentiation both *in vivo* and *in vitro*. They also suggest that the few mature B cells found in *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* and *c-myc$^{fl/fl}$; mb-1$^{cre/+}$; rosa26$^{gfp/gfp}$* mouse spleens are not generated in the absence of c-Myc.

The other animal model, the inducible *c-myc$^{fl/fl}$;mx-cre$^+$* mice, allowed us to study the c-Myc requirement at later stages of B cell development. We induced *c-myc* deletion with pIpC in the BM of 5- to 8-week-old *c-myc$^{fl/fl}$; mx-cre$^+$* mice. Five days after the last injection, mice were sacrificed and B

cell differentiation was studied by flow cytometry. We observed that homozygous *cmyc$^{fl/fl}$; mx-cre^{+}* mice had an acute reduction (>3.5-fold) in absolute B cell numbers, as well as a reduction in both B220^{+}IgM^{-} cells (Fractions A to D) and B220^{lo}IgM^{+} cells (Fraction E) compared to *c-myc$^{fl/fl}$; mxcre^{-}* controls (Fig. R3f).

Taken together, all these data suggest that the *c-myc* gene is required for B cell development.

4.2.2. c-Myc-deficient immature B cells die by apoptosis

One of the most studied functions of c-Myc is its role in apoptosis. The first observation that implicated c-Myc in apoptosis was in murine fibroblasts, in which *c-myc* overexpression in low serum conditions led to programmed cell death (Evan et al., 1992) (Hueber et al., 1997) (Thompson, 1998). Several studies correlated deregulation of *c-myc* expression with apoptosis in a number of cell lines. In human and murine B lymphomas, apoptosis induced through the BCR correlates with negative modulation of *c-myc* levels (Brunner T et al., 1995). In immature B cell lines , BCR engagement leads to a drop in c-Myc protein, eventually leading to apoptosis (Wu et al., 1996)

The reduction in B220^{+}IgM^{-} cells in *c-myc$^{fl/}$*

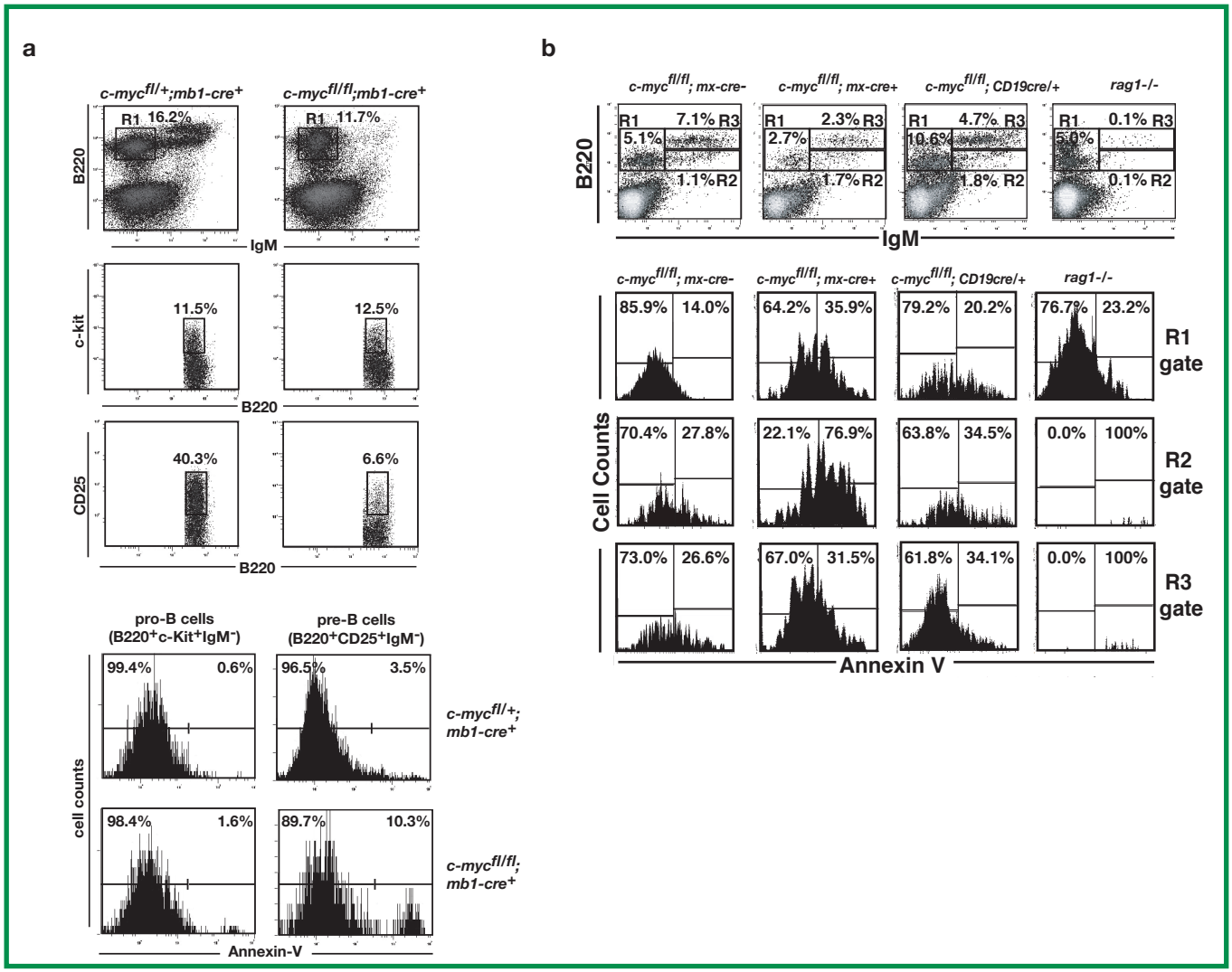

Fig.R5. c-Myc-deficient immature B cells die by apoptosis. (a) Representative annexin V staining. Four-week-old *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mouse BM suspensions were stained with antibodies to B220, IgM, c-kit to define pro-B cells (B220^{+}c-kit^{+}IgM^{-}) and CD25 for pre-B cells (B220^{+}CD25^{+}IgM^{-}). Cells were further stained with annexin V to detect apoptotic cells (annexin V^{+}) within pro- and pre-B populations. (b) Annexin V staining of BM cells from *c-myc$^{fl/fl}$; mx-cre^{+}* mice and control littermates. B220^{+}IgM^{-} (pro- and pre-B cells), B220^{lo}IgM^{+} (immature B cells) and B220^{hi} IgM^{+} (mature B cells) populations were gated and analysed for annexin V-positive cells.

fl; *mb-1*$^{cre/+}$ could be attributed to three possible reasons: an increase in apoptosis levels of c-Myc-deficient B lymphocytes, a decrease in the proliferation of these cells, or a combination of both. To determine whether c-Myc-deficient B lymphocytes died by apoptosis, we used annexin-V staining in pro- and pre-B cells from homozygous *c-myc*$^{fl/fl}$; *mb-1*$^{cre/+}$ and heterozygous *c-myc*$^{fl/+}$; *mb-1*$^{cre/+}$ littermate control mice. B220^{+}IgM^{-} cells can be subdivided into pro-B cells (B220^{+}IgM^{-} c-kit^{+}) and pre-B cells (B220^{+}IgM^{-}CD25^{+}). Our data showed a slight increase in annexin-V positive cells in both c-Myc-deficient pro-B (0.6% vs. 1.6%; 2.6-fold increase) and pre-B cells (3.5% vs. 10.3%; 2.9-fold increase) (Fig. R5a).

Annexin-V staining also revealed an increase in the relative numbers of apoptotic B220^{lo}IgM^{-} (14.0% vs. 35.9%) and B220^{lo}IgM^{+} (27.8% vs. 76.9%) cells in the BM of *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$ mice compared to controls (Fig. R5b). Consistent with previous observations in *c-myc*$^{fl/fl}$; *cd19*$^{cre/+}$ mice (de Alboran et al., 2001), B220^{hi}IgM^{+} mature B lymphocytes from *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$ mice (26.6% vs. 31.5%) survived in the absence of *c-myc*.

These data suggested that c-Myc is required for the survival of pro-, pre- and immature B cells and strengthen our hypothesis that c-Myc is required for B cell differentiation.

4.2.3. B lymphocytes are able to proliferate in the absence of c-Myc

By regulating cell cycle genes, c-Myc promotes proliferation in many cell types, including B lymphocytes (Bernard and Eilers, 2006). To study proliferation in c-Myc-deficient B lymphocytes, we performed *in vivo* BrdU incorporation analysis in homozygous *c-myc*$^{fl/fl}$; *mb-1*$^{cre/+}$ mice and heterozygous *c-myc*$^{fl/+}$; *mb-1*$^{cre/+}$ littermate controls. We sorted the B220^{+}IgM^{-} population, stained cells with anti-BrdU antibody and assessed proliferation by FACS analysis. c-Myc-deficient pro- and pre-B lymphocytes showed decreased proliferation compared to the same population in WT mice (7.9% vs. 22.9%; 2.8-fold decrease) (Fig. R6a). Again, similar results were observed in B220^{+}IgM^{-} lymphocytes (18% vs. 34%, 1.8- fold decrease) from *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$(Fig. R6b).

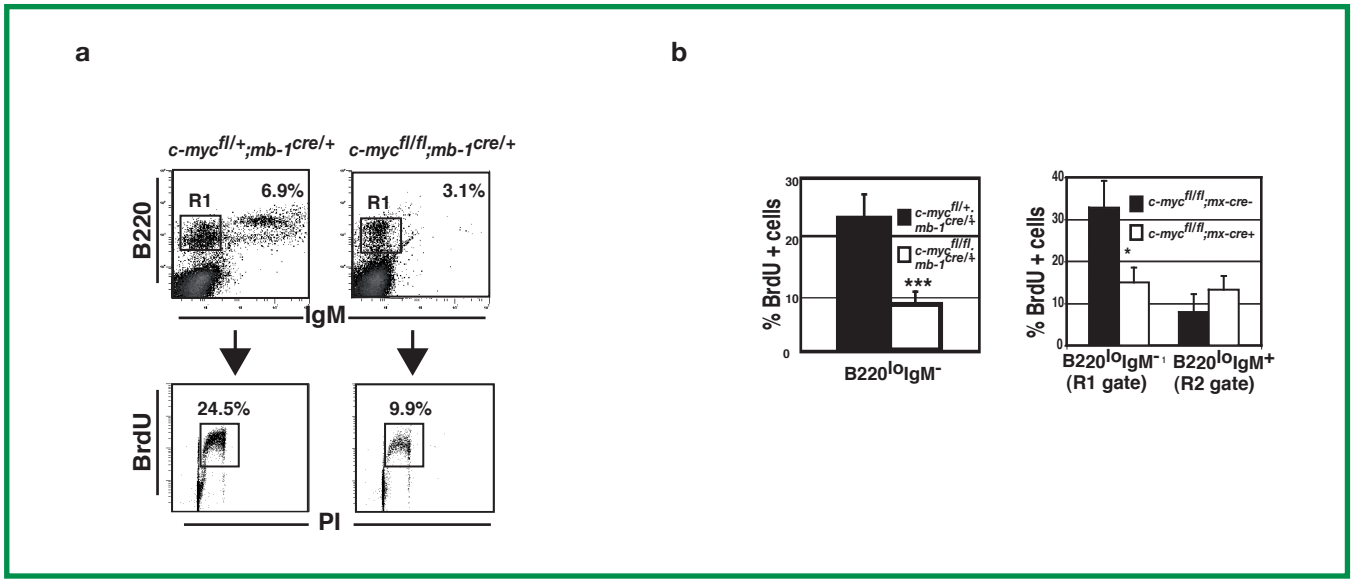

Fig. R6. B lymphocytes proliferate in the absence of c-Myc. (a) Five- to 10-week-old animals received BrdU injections to measure B cell proliferation by BrdU incorporation. B220^{+}IgM^{-} cells from homozygous and control mice were sorted and stained with an anti-BrdU antibody and PI to identify proliferating cells. (b) (left) Scheme showing the percentage of BrdU-positive cells in *c-myc*$^{fl/+}$; *mb-1*$^{cre/+}$(black bars, n = 5) and *c-myc*$^{fl/fl}$; *mb-1*$^{cre/+}$ (open bars, n = 4). P<0.001. (right) As above, using *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$mice (open bars) and control littermates (black bars).

These results show that c-Myc-deficient pro- and pre-B lymphocytes are able to proliferate in the absence of *c-myc*.

4.2.4. V(D)J recombination is impaired in c-Myc-deficient B lymphocytes

B cell development in the BM takes place in successive stages during which the IgH V (variable), D (diversity) and J (joining) gene segments are rearranged in an ordered and sequential fashion. First D_H-to-J_H segments rearrange, which are followed by V_H-to-DJ_H rearrangements. To determine whether the impaired B cell differentiation observed in c-Myc-deficient lymphocytes was characterised by defective V(D)J recombination, we sorted $B220^+IgM^-$ BM cells from homozygous *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* and control littermates. Genomic PCR with specific primers for rearranged D_H-J_H, $V_{Hproximal}$-DJ_H and $V_{Hdistal}$-DJ_H segments showed that in the case of c-Myc-deficient $B220^+IgM^-$ cells, all types of rearrangements were reduced compared with control cells (Fig. R7). These results indicated that c-Myc-deficient pro- and pre-B cells have impaired V(D)J recombination.

4.3. Gene expression profile in c-Myc-deficient B cells

We next wanted to identify a molecular mechanism that could explain the requirement of c-Myc for B cell differentiation in *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice. We analysed expression of several B cell-specific genes in sorted $B220^+IgM^-$ cells from both homozygous *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* and control *c-myc$^{fl/+}$; mb-1$^{cre/+}$* mice (Fig. R8a, b). As predicted, *c-myc* expression was downregulated in pro- and pre-B cells from homozygous mice, as compared to WT levels The expression levels of transcription factors E2A, EBF and Pax5 were considerably reduced in c-Myc-deficient pro- and pre-B cells compared to WT cells (Fig R8a). In the case of *pax5*, expression levels were reduced not only for this transcription factor, but also for its well-known target genes such as *blnk, n-myc, Igα* and *Igβ* (Nutt et al., 1998) (Fig. R8a).

As described above (introduction section 1.4), the E2A/EBF/Pax5 transcription factor network is involved in the specification and commitment of B cell progenitors into the B lineage (Bartholdy and

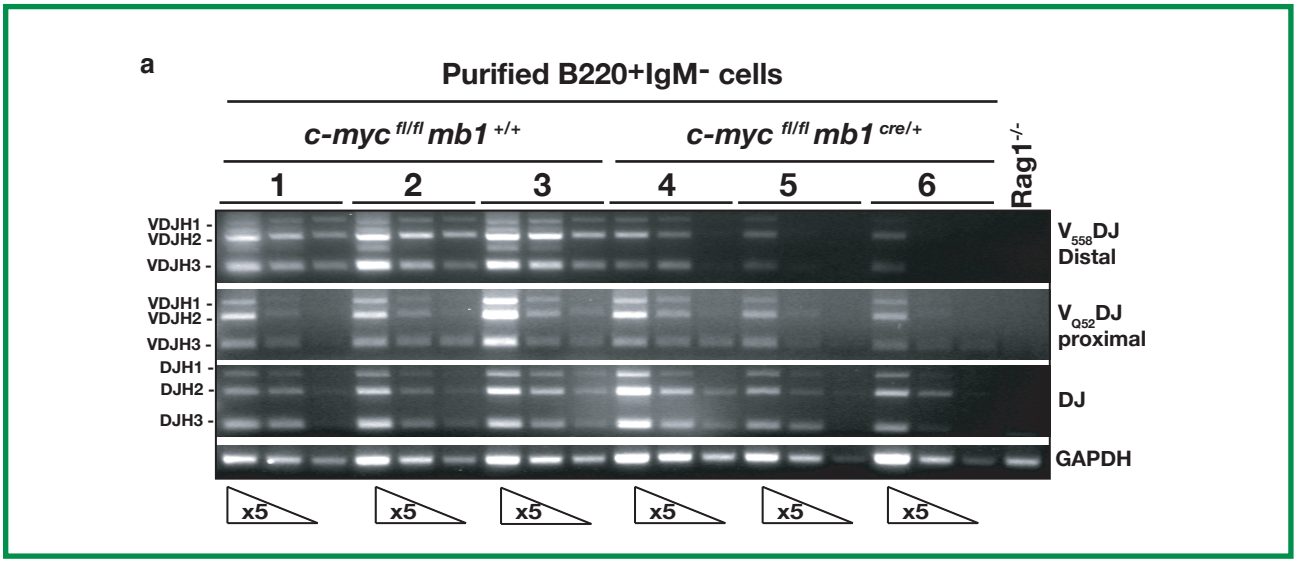

Fig. R7. V(D)J recombination is impaired in c-Myc-deficient B lymphocytes. PCR analysis of IgH rearrangement in genomic DNA from sorted $B220^+IgM^-$ cells from *c-myc$^{fl/fl}$; mb-1$^{+/+}$* and *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice. Rearrangements were analysed with primers that detect V_HJ558-to-DJ_H, V_HQ52-to-DJ_H and D_H-to-J_H rearranged segments.

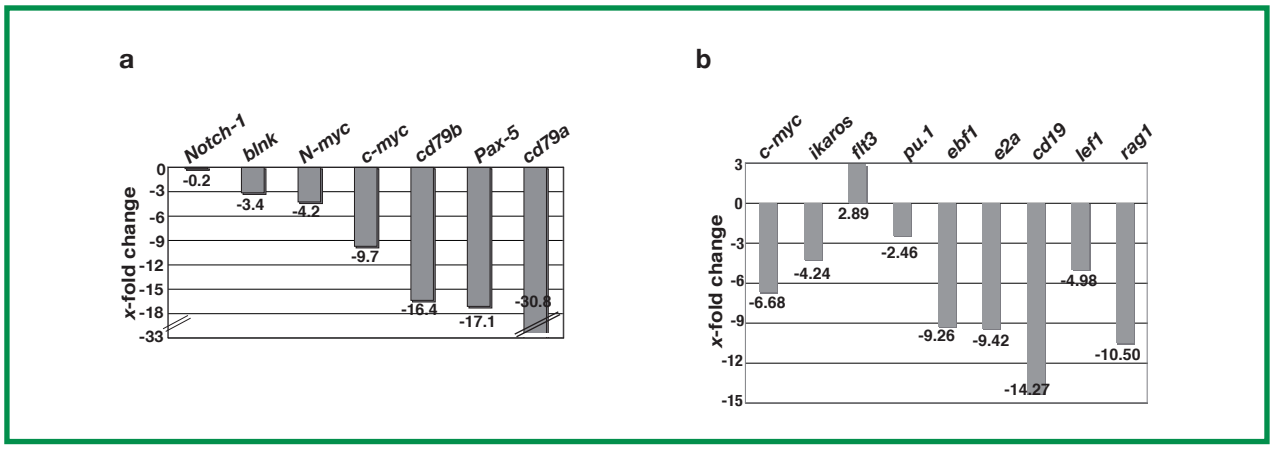

Fig. R8. c-Myc function in B cell differentiation. (a) B220^{+}IgM^{-} cells from *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* and littermate control mice were sorted, and we synthesised cDNA and used qRT-PCR to study the expression of a battery of B cell-specific genes. Data represent at least three independent experiments.

Matthias, 2004) (Medina et al., 2004); (Roessler and Grosschedl, 2006) (Fuxa and Skok, 2007). Our results show that c-Myc-deficient pro- and pre-B cells have downregulated expression of key transcription factors involved in the B cell differentiation program.

4.4. c-Myc deficient pro-B cells express lower surface levels of CD19 and pre-BCR than WT cells

Pax5$^{-/-}$ pro-B cells lack CD19 expression (Nutt et al., 1997). CD19 is a direct Pax5 target gene, and measurement of CD19 surface levels by FACS is a good tool for measurement of Pax5 activity (Nutt et al., 1998).

We stained total BM cells and used FACS to detect surface expression of CD19 in B220^{+}IgM^{-} cells from *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice, control littermates, and from *rag1$^{-/-}$* and *E*μ*-myc* transgenic mice as additional controls (Fig. R9a). *rag1$^{-/-}$* mice have a B cell block at the pro-B cell stage similar to that of the *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice, and *E*μ*-myc* transgenic mice constitutively express a *c-myc* transgene under the control of the Ig heavy chain enhancer *E*μ. CD19 surface levels were greatly reduced in c-Myc-deficient pro- and pre-B cells (7.2% vs. 68.4%; 9.5-fold reduction) compared to wild-type**,** as well as to *rag1$^{-/-}$* and *E*μ*-myc* transgenic mice. This confirm the results obtained in the previous section, in which we found that CD19 transcriptional levels were downregulated in B220^{+}IgM^{-} cells from *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice compared to wild-type mice.

Pax5-deficient pro-B cells do not express the pre-BCR on their surface (Thevenin et al., 1998), (Schebesta et al., 2002), as these cells do not express the signal-transducing molecules Igα and Igβ, components of the pre-BCR. To study pre-BCR surface levels in c-Myc-deficient B cells, we used an antibody that recognises a conformational epitope on the surrogate light chain-Igμ complex of the pre-BCR, and stained cells from *c-myc$^{fl/fl}$; mb-1$^{cre/+}$*, control littermates, from *rag1$^{-/-}$* and *E*μ*-myc* transgenic mice. We found that c-Myc-deficient cells expressed lower surface levels of the pre-BCR (0.1% vs. 0.3%; 3-fold decrease) than control cells (Fig. R9b).

These results show that c-Myc-deficient B220^{+}IgM^{-} cells have lower surface expression levels of CD19 and pre-BCR.

4.5. c-Myc-deficient B cells do not give rise to other lineages when cultured with appropriate cytokines

When they are stimulated with appropriate cytokines, Pax5-deficient pro-B cells are able to differentiate *in vitro* into functional macrophages, osteoclasts, dendritic cells, granulocytes and natural killer cells (Nutt et al., 1999). Ectopic expression

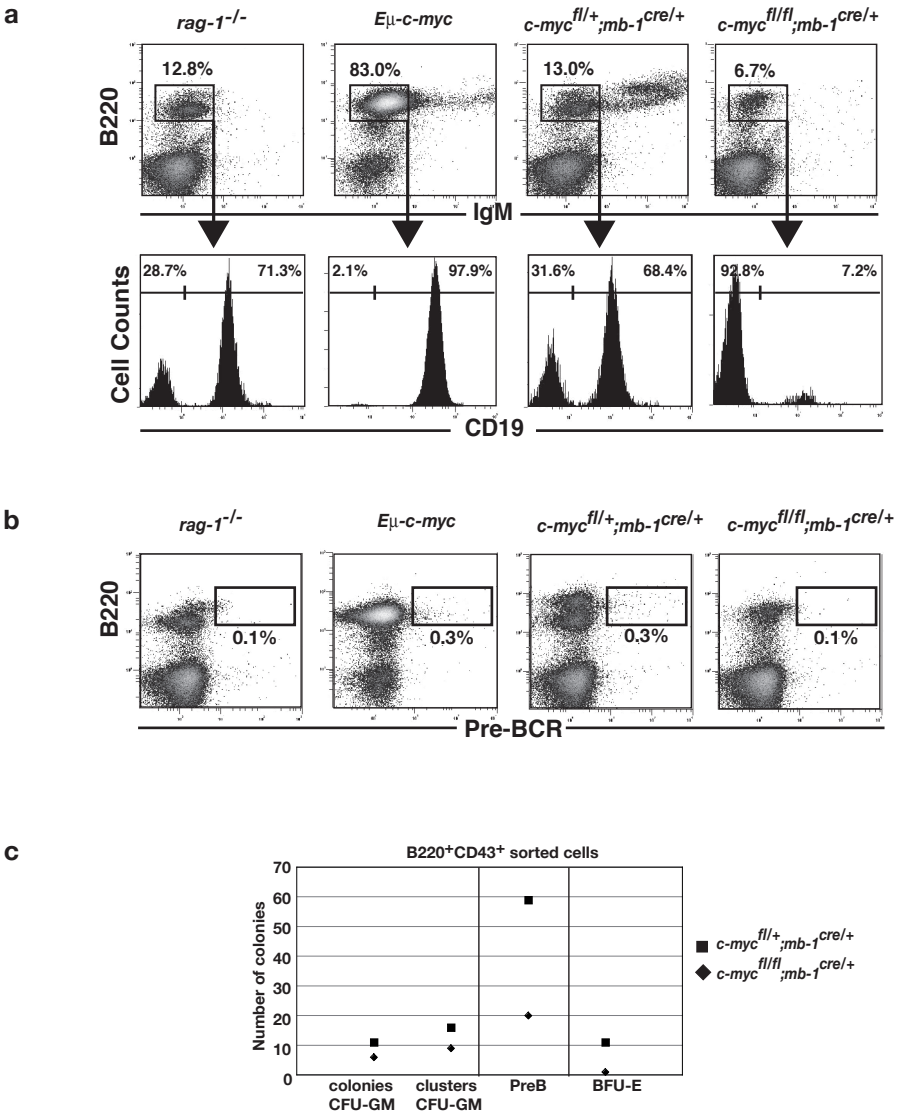

Fig. R9. c-Myc-deficient pro- and pre-B cells express lower surface levels of CD19 and pre-BCR than WT cells and do not generate cells of other lineages when cultured with appropriate cytokines. (a) Lower CD19 expression levels in c-Myc-deficient B cells. BM of the indicated genotypes was analysed by flow cytometry, and CD19 expression is displayed for pro- and pre-B cells ($B220^+IgM^-$). (b) Analysis of pre-BCR expression. Representative FACS analysis of pre-BCR surface expression are shown for *rag1*$^{-/-}$, *Eμ-c-myc*, *c-myc*$^{fl/+}$*; mb-1*$^{cre/+}$ and *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice. *Rag1*$^{-/-}$ and *Eμ-c-myc* mice are shown as negative and positive controls, respectively. (c) Methylcellulose colony assays. The lymphoid, myeloid and erythroid colony forming-activity of $B220^+CD43^+$ BM cells from *c-myc*$^{fl/+}$*; mb-1*$^{cre/+}$ and *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice was quantified as the capacity to form colony (CFU)- or burst-forming units (BFU) of the different lineages in semisolid methylcellulose media.

of C/EBPα and GATA factors strongly promote *in vitro* macrophage differentiation and myeloid colony formation of Pax5$^{-/-}$ pro-B cells (Heavey et al., 2003). In another study, Pax5-deficient B cell progenitors transfer into Rag2-deficient mice provided long-term reconstitution of T cell development (Rolink et al., 1999).

To study whether c-Myc-deficient B cells were able to give rise to different lineages *in vitro*, we sorted $B220^+CD43^+$ cells from *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ and control littermates and cultured them in methylcellulose-based media supplemented with cytokines and growth factors, and quantified colony-forming cells of the erythroid, granulocyte/macrophage and B lineages. We found

that c-Myc-deficient B cells only gave rise to cells of the B lineage and were three times less efficient than WT cells (Fig. R9c). These data indicate that c-Myc-deficient immature B lymphocytes are committed to the B lineage and cannot give rise to cells of other haematopoietic lineages when cultured *in vitro* with appropriate cytokines.

4.6. Ectopic expression of Pax5 in c-Myc-deficient B lymphocytes

To study whether Pax5 expression in c-Myc-deficient cells was sufficient to rescue the block in B cell differentiation, we used the $Ik^{neo/+}$ conditional mouse model that expresses the *pax5* gene from the endogenous *ikaros* promoter (Souabni et al., 2002).

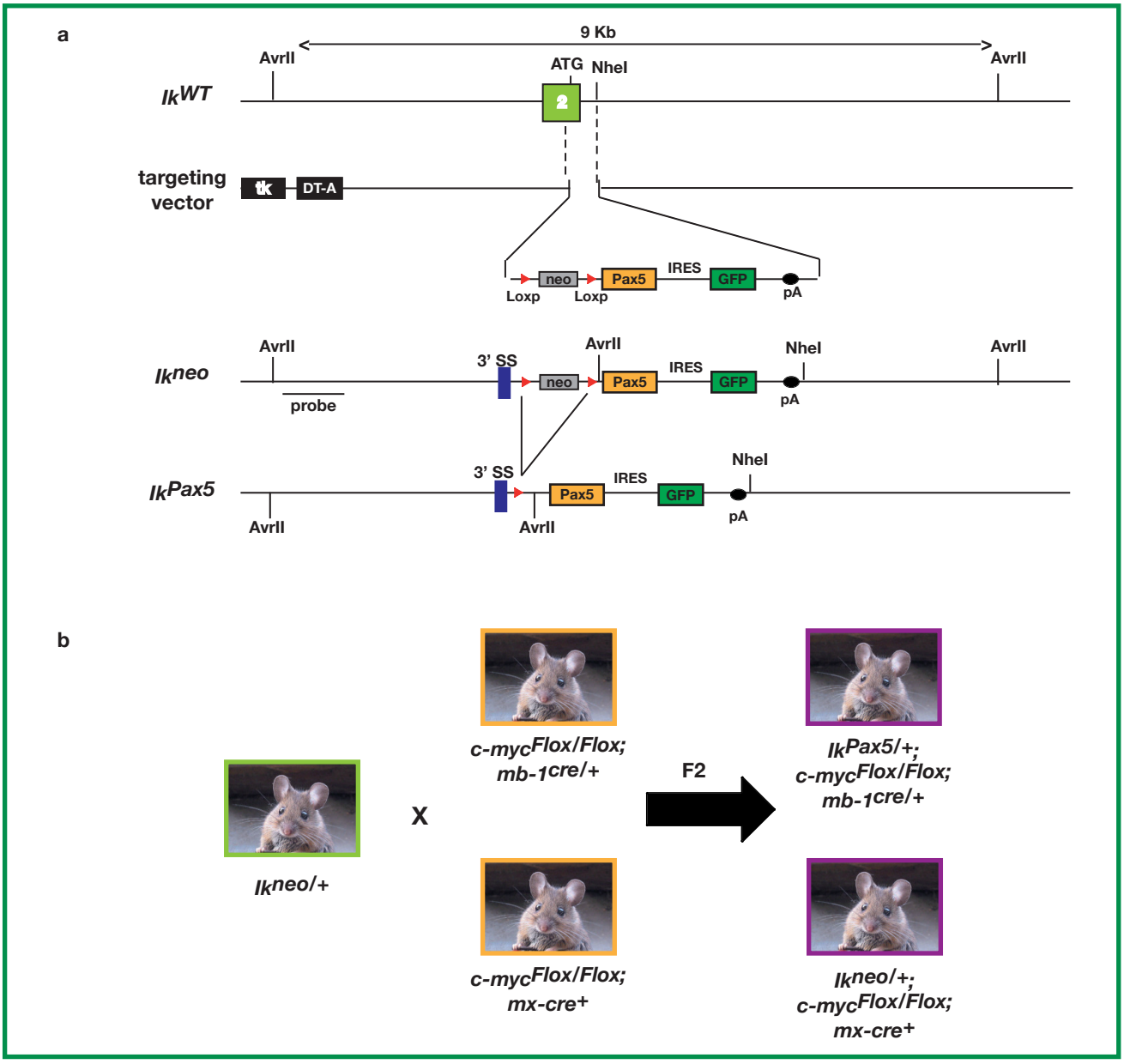

Fig. R10. Generation of *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ and *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice. (a) Insertion of a *pax5* minigene into the *Ikaros* locus. The conditional *Ik*^{neo} allele was generared by inserting human *pax5* cDNA together with a floxed neomycin (*neo*) resistance gene upstream of the translation start codon in *Ikaros* exon 2. *Pax5* cDNA was linked via an IRES site to a GFP gene and the 3' polyadenylation (pA) region of the rabbit β-*globin* gene. Red arrowheads: *loxP* sites. (b) Generation of *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ and *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice. *Ik*^{neo} mice were bred with *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ or *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ to generate two different mouse models for the study of Pax5 expression in c-Myc-deficient B cells.

4.6.1. Generation of *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* and *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}*

The *Ikaros* (*Ik*) gene is widely expressed throughout the haematopoietic system, including HSC and their committed progenitors (Kelley et al., 1998). To study the effect of Pax5 on the development of all haematopoietic lineages, we used a heterozygous knock-in mouse carrying *pax5* gene under the control of the *Ikaros* locus (Souabni et al., 2002). The conditional *Ik$^{neo/+}$* mouse was constructed by inserting a *pax5* mini-gene linked via an IRES sequence to a *GFP* gene, together with a floxed neomycin (*neo*) resistance gene, into the *Ik* locus (Fig. R10a). When these mice are bred with mice bearing a stage-specific *Cre* recombinase gene, the *neo* resistance gene is deleted and there is expression of Pax5 and GFP.

To express Pax5 in c-Myc-deficient cells, we bred *Ik$^{neo/+}$* mice with either *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* or *c-myc$^{fl/fl}$; mx-cre^{+}* mice (Fig. R10b). The F2 generations generated homozygous *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* and control *Ik$^{neo/+}$; c-myc$^{fl/+}$; mb-1$^{cre/+}$*, or homozygous *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* and control *Ik$^{neo/+}$; c-myc$^{fl/+}$; mx-cre^{+}* mice. We generated two mouse models that express *pax5* in B lymphocytes, although the onset of expression differs between the two models. In the case of *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice, Pax5 is expressed in a constitutive, specific manner in c-Myc-deficient pro-B cells. For Pax5 expression in *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* mice, Cre recombinase must be activated by pIpC; once Cre is activated, Pax5 is expressed from the early precursors in the BM to the more mature B cells in spleen.

4.6.2. Ectopic expression of Pax5 in *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice does not rescue B cell differentiation

To determine whether Pax5 expression in c-Myc-deficient pro- and pre-B cells could rescue the developmental block observed in *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice, we obtained total BM from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* and control *Ik$^{neo/}$; c-myc$^{fl/+}$; mb-1$^{cre/+}$* mice and stained cells with the B220, IgM, CD19 and CD43 surface markers to allow us to distinguish B cell subsets. We used both young mice (2-3 weeks of age) and adult mice, which showed no substantial differences. Pax5 expression in these cells was insufficient to overcome the developmental block; neither B220^{+}IgM^{+} nor B220^{+}CD43^{-} cells were found in the BM of homozygous *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice (Fig.R11a). In addition, we found the same reduction in the B220^{+}CD19^{+} population as previously observed in *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice. Absolute numbers also reflected a severe reduction in B cells (B220^{+}; 8-fold decrease) and in B220^{+}IgM^{+} cells (4.8-fold decrease) compared to age-matched controls (Fig. R11b). We also analysed CD19 surface levels in B220^{+}IgM^{-} cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* and control littermates, and found pro- and pre-B c-Myc-deficient lymphocytes had reduced CD19 surface expression levels (79.5% vs. 13.0%: 6-fold decrease) (Fig. R11c). These results indicate that *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb -1$^{cre/+}$* mice show the same developmental blockade as *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice.

IgM-depleted BM cells from *c-myc$^{fl/+}$; mb-1$^{cre/+}$* mice, *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice and *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice were *in vitro* cultured and differentiation was analysed at days 3 and 7 (see section 4.4). We found that BM cells from neither *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* nor *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice gave rise to B220^{+}IgM^{+} or B220^{+}CD19^{+} *in vitro* (Fig. R12). These results show that BM cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice cannot differentiate into IgM^{+} B cells *in vitro*, confirming our earlier results in section 4.4.

4.6.3. Transcriptional expression levels of *pax5, ebf, e2a* and *c-myc* in pro- and pre-B cells from *Ik$^{neo/+}$; cmyc$^{fl/fl}$; mb-1$^{cre/+}$* mice

To test whether the failure to rescue B cell differentiation upon expression of *pax5* in c-Myc-deficient B cells in *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice was due to inefficient Pax-5 expression in these animals, we measured the transcription levels of *pax5* and *c-myc*. We also analysed the expression of the B cell-specific factors *ebf1* and *e2a*, to determine whether the blockade was due solely to *pax5* and *c-myc* deficiency,

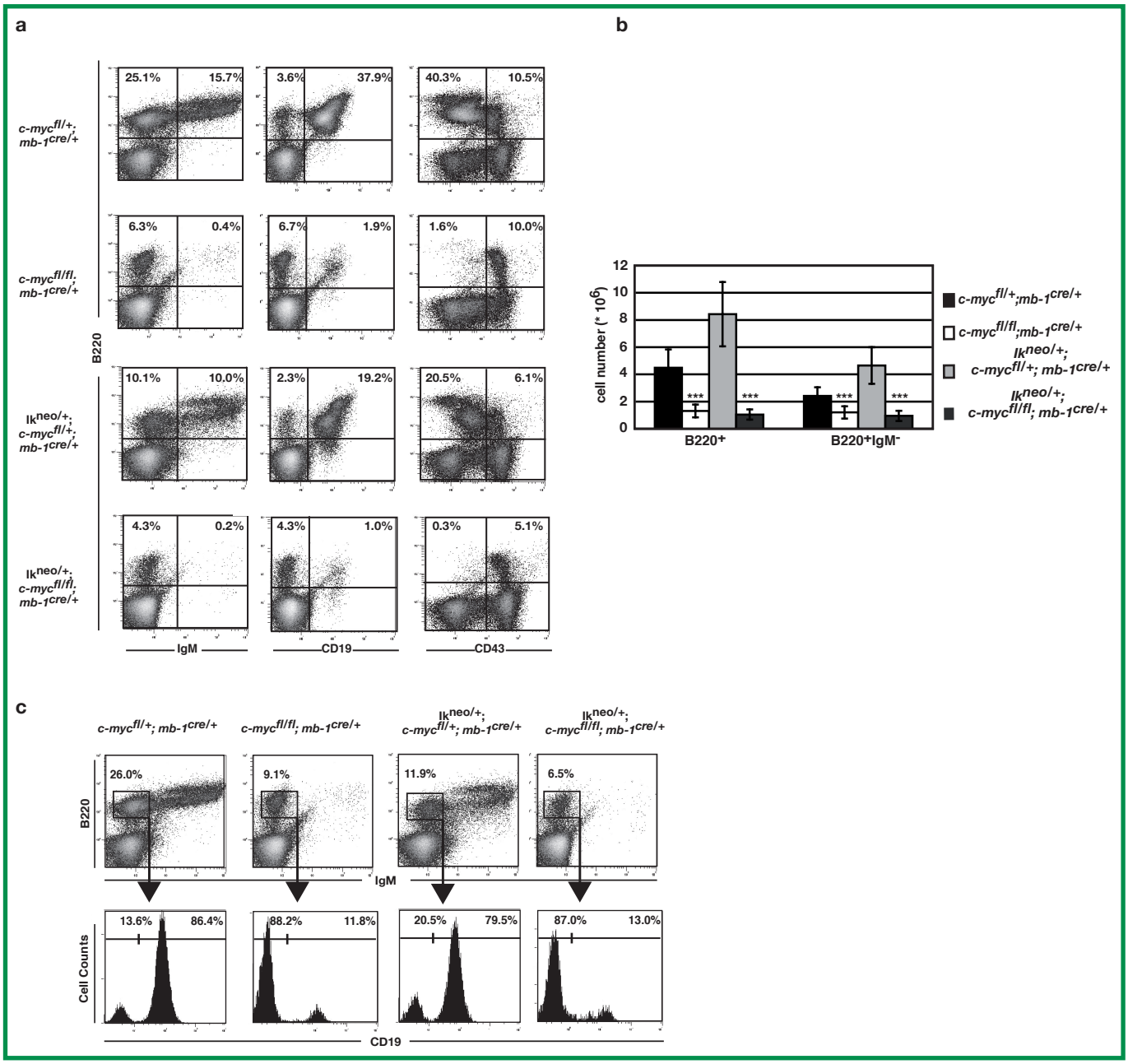

Fig. R11. Impaired B lymphopoiesis in *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice. (a) Representative flow cytometric analysis of surface expression of B220, IgM, CD19 and CD43 to study B cell development in *c-myc$^{fl/+}$; mb-1$^{cre/+}$*, *c-myc$^{fl/+}$; mb-1$^{cre/+}$*, *Ik$^{neo/+}$; c-myc$^{fl/+}$; mb-1$^{cre/+}$* and *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice. (b) Absolute numbers of B cells (B220$^+$) and pro- and pre-B cells (B220$^+$IgM$^-$) in the BM of mice of the genotypes in (a). P <0.001. (b) CD19 surface expression in pro- and pre-B cells from the same genotypes as above. Stainings are representative of at least three independent experiments.

or if downregulation of *ebf1* and *e2a* was also involved. B220$^+$IgM$^-$ cells from control *c-myc$^{fl/+}$; mb-1$^{cre/+}$* and homozygous *c-myc$^{fl/fl}$; mb-1$^{cre/+}$* and *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* mice were sorted and cDNA was synthesised for qRT-PCR with specific primers for these genes.. We found that transcriptional levels of *c-myc, pax5, ebf1* and *e2a* were downregulated (by 90.2-, 2.5-, 67.3-, and 143-fold, respectively) in B220$^+$IgM$^-$ cells from homozygous *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$* compared to *c-myc$^{fl/+}$; mb-1$^{cre/+}$* mice (Fig. R13).

Overall, these data indicates that B220$^+$IgM$^-$ cells from homozygous *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mb-1$^{cre/+}$*, are unable to express *pax5* at wild-type levels upon activation of *cre* recombinase. In addition, transcriptional levels of *ebf* and *e2a* remain downregulated compared to control cells.

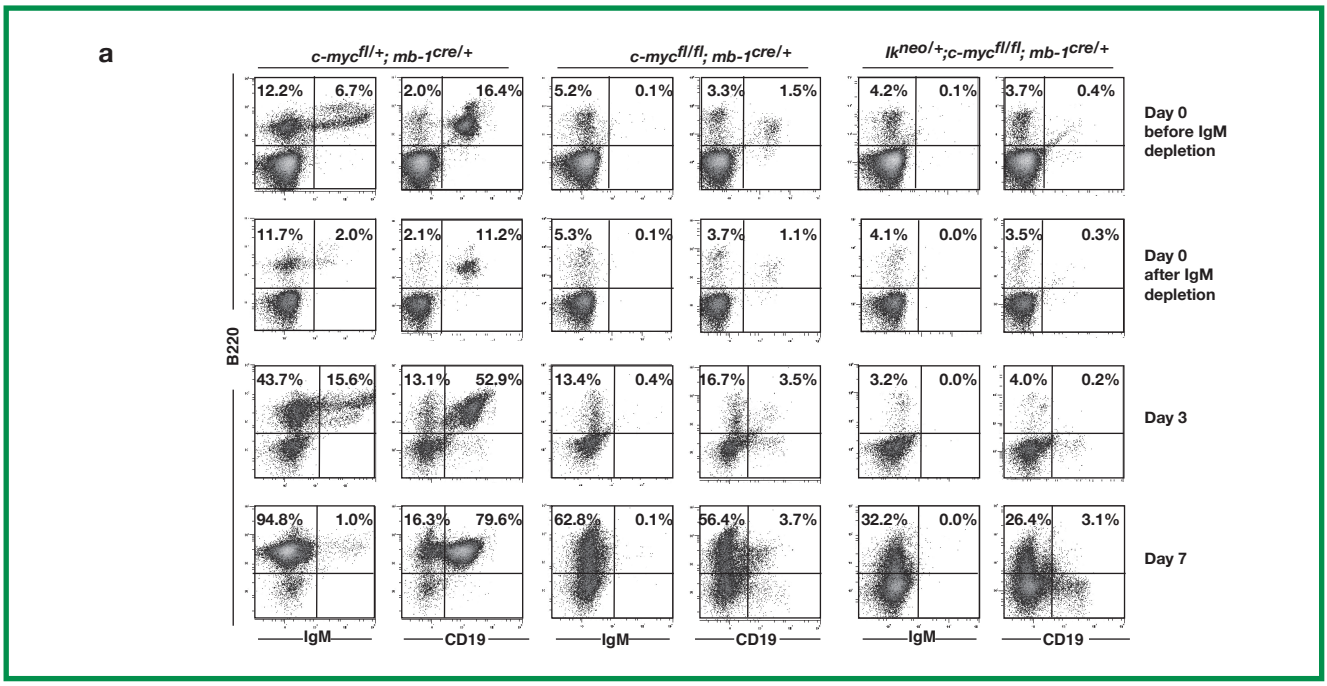

Fig. R12. BM cells from *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice cannot differentiate into IgM$^+$ cells *in vitro*. Cells were cultured as described (see Materials and Methods) and analysed by flow cytometry at days 3 and 7 of culture for B220, IgM and CD19 expression.

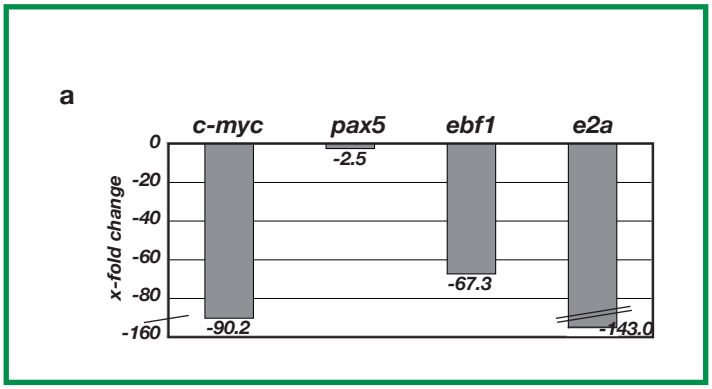

Fig. R13. Transcriptional levels of *pax5, ebf, e2a* and *c-myc* in pro- and pre-B cells from *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice. B220$^+$IgM$^-$ cells from *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice and *cmyc*$^{fl/+}$*; mb-1*$^{cre/+}$ controls were sorted; we then synthesised cDNA and used qRT-PCR to study the expression of Pax5, EBF and E2A transcription factors.

4.6.4. *In vivo* rescue of B cell differentiation in *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^+$ mice

To determine whether *pax5* expression in early progenitors could rescue B lymphopoiesis in c-Myc-deficient mice, we used 4- to 6-week-old animals that were inoculated three times with 150 µg pIpC and sacrificed three days after the last injection. Cell surface marker expression analysis (see section 4.7.2) showed substantial rescue when we compared the phenotype of *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^+$ with homozygous *c-myc*$^{fl/fl}$*; mx-cre*$^+$ knockout mice (Fig. R14a). Examination of B220 vs. CD43 staining indicated that although relative B220$^+$CD43$^+$ cell numbers remained similar, there was an increase in the B220^{lo}CD43$^-$ population in Hardy Fractions D and E (3.1% vs. 0.8%; 3.9-fold increase). A similar increase was observed in the B220$^+$IgM$^-$ population comprised by Fractions A to D (8.0% vs. 4.9%; 1.6-fold increase) and in the B220^{lo}IgM$^+$ population, which corresponds to Fraction E (2.1% vs. 0.8%; 2.6-fold increase). Comparison of absolute cell numbers between homozygous *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^+$ and *c-myc*$^{fl/fl}$*; mx-cre*$^+$ knockout mice (Fig. R14b) reflected a more acute increase in B220$^+$IgM$^-$ cells (1.7 x 10^6 vs. 0.7 x 10^6; 2.4-fold increase) and in B220^{lo}IgM$^+$ (0.5 x 10^6 vs. 0.1 x 10^6; 5-fold increase). These results suggest that ectopic Pax5 expression in c-Myc-deficient BM from haematopoietic precursors enables rescue of B cell differentiation *in vivo*.

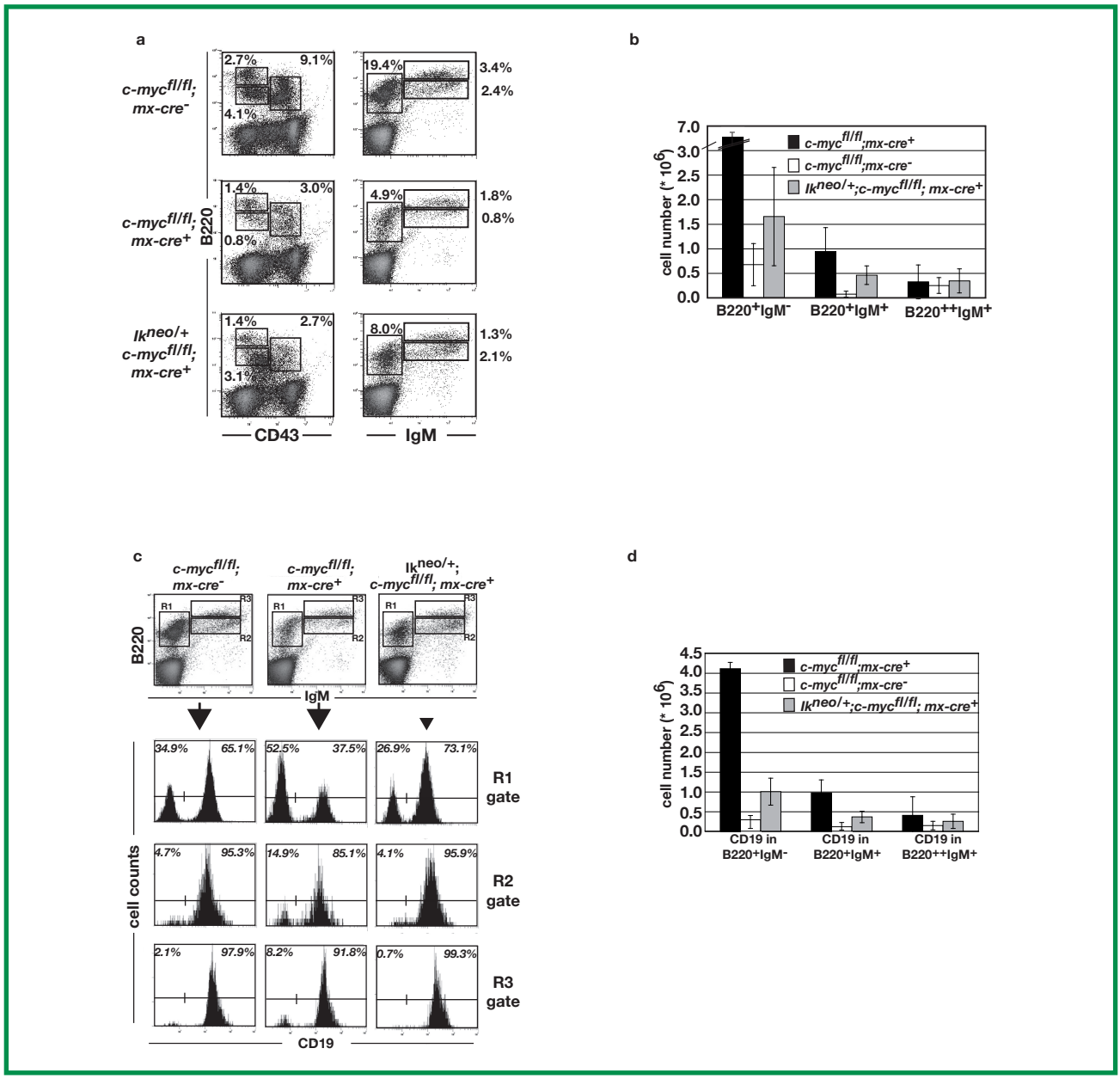

Fig. R14. ***In vivo*** **rescue of B cell differentiation in** $Ik^{neo/+}$**;** $c\text{-}myc^{fl/fl}$**;** $mx\text{-}cre^{+}$ **mice.** (a) BM cells from 4- to 6-week-old pIpC-inoculated mice of the indicated genotypes were stained with the antibodies indicated in Fig. R12 to study B cell development. (b) Absolute numbers of $B220^{+}IgM^{-}$ (pro- and pre-B cells), $B220^{lo}IgM^{+}$ (immature B cells) and $B220^{hi}IgM^{+}$ (mature B cells) from control *c--*$myc^{fl/fl}$*;* $mx\text{-}cre^{-}$ (n = 4), homozygous $c\text{-}myc^{fl/fl}$*;* $mx\text{-}cre^{+}$(n = 4) and $Ik^{neo/+}$*;* $c\text{-}myc^{fl/fl}$*;* $mx\text{-}cre^{+}$(n = 5) mice. (c) CD19 expression in the different B cell subsets. CD19 expression is displayed for $B220^{+}IgM^{-}$, $B220^{lo}IgM^{+}$ and $B220^{hi}IgM^{+}$ cells. (d) Absolute numbers of $CD19^{+}$ cells in the different B cell subpopulations from $c\text{-}myc^{fl/fl}$*;* $mx\text{-}cre^{-}$ (n=3), $c\text{-}myc^{fl/fl}$*;* $mx\text{-}cre^{+}$ (n = 3) and $Ik^{neo/+}$*;* $c\text{-}myc^{fl/fl}$*;* $mx\text{-}cre^{+}$(n = 4) mice.

We also analysed CD19 surface expression in $B220^{+}IgM^{-}$, $B220^{lo}IgM^{+}$ and $B220^{hi}IgM^{+}$ cells to assess Pax5 activity (Fig. R14c). $B220^{+}IgM^{-}$ cells from $Ik^{neo/+}$*;* $c\text{-}myc^{fl/fl}$*;* $mx\text{-}cre^{+}$ mice rescued CD19 expression as compared to homozygous $c\text{-}myc^{fl/fl}$*;* $mx\text{-}cre^{+}$ mice, whereas we found no differences in the three mouse genotypes in surface CD19 expression on $B220^{lo}IgM^{+}$ and $B220^{hi}IgM^{+}$ cells. This concurs with our prediction, as CD19 expression begins in fraction B and remains throughout development until the mature B cell stage (Nagasawa, 2006). The increase in surface CD19 expression on $B220^{+}IgM^{-}$ cells in $Ik^{neo/+}$*;* $c\text{-}myc^{fl/fl}$*;* $mx\text{-}cre^{+}$ compared to $c\text{-}myc^{fl/fl}$*;* $mx\text{-}cre^{+}$ mice was also reflected by absolute cell numbers (1.0 x 10^6 vs. 0.3 x 10^6; 3.3-fold increase) (Fig. R14d). These findings indicate that Pax5 expression in c-Myc-deficient pro-

and pre-B lymphocytes is sufficient to induce CD19 surface expression in these cells.

4.6.5. c-Myc-deficient B cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* mice can differentiate into IgM^{+} B cells *in vitro*

To study whether Pax5 expression induced not only survival of c-Myc-deficient B lymphocytes, but also B cell differentiation, we depleted IgM-positive cells from BM (see section 4.4). Young adult (4- to 6-week-old) mice were inoculated three times with 400 µg pIpC and sacrificed three days after the last dose. We cultured IgM-depleted BM from *c-myc$^{fl/fl}$; mx-cre^{-}*, *from c-myc$^{fl/fl}$; mx-cre^{+}*, from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{-}* and from *Ik$^{neo/+}$; c--myc$^{fl/fl}$; mx-cre^{+}* mice for 3 days and evaluated IgM^{+} cells by FACS analysis. We identified B220^{+}IgM^{+} cells in homozygous *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* mice, whereas no cells of this type were generated in homozygous *c-myc$^{fl/fl}$; mx-cre^{+}* mice (Fig. R15a). Finally, *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* mice were able to generate B220^{+}CD19^{+} cells, which were not present in *c-myc$^{fl/fl}$; mx-cre^{+}* mice (Fig. R15a). These results show that *pax5* expression in c-Myc-deficient B cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* mice is sufficient to overcome the B cell differentiation impairment *in vitro* and suggests that it does so *in vivo* too.

4.6.6. Transcriptional expression levels of *pax5, ebf, e2a* and *c-myc* in immature B cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* mice

To analyze the transcriptional levels of the *pax5, ebf*, *e2a* and *c-myc* genes, we sorted B220^{+}IgM^{-} cells from pIpC-treated control *c-myc$^{fl/fl}$; mx-cre^{-}*, homozygous *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* and *c-myc$^{fl/fl}$; mx-cre^{+}* mice. We synthesised cDNA and used qRT-PCR to study *pax5, ebf, e2a* and *c-myc* expression.

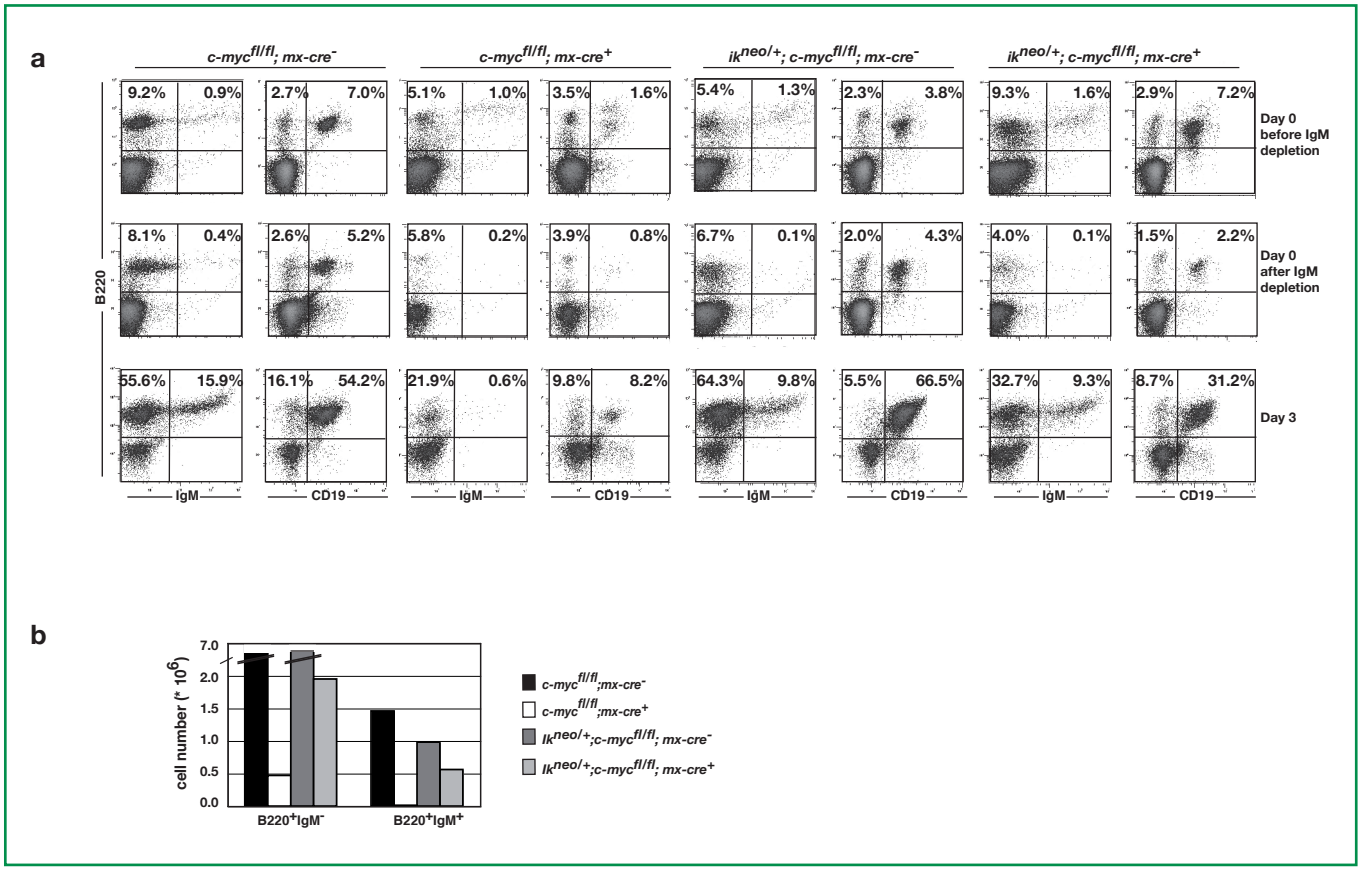

Fig. R15. ***In vitro*** **rescue of B cell differentiation in *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* mice.** (a) BM cells of the indicated genotypes were cultured *in vitro* and differentiation was analysed by flow cytometry at day 3 of culture for B220, IgM and CD19 expression. (b) Absolute numbers of B220^{+}IgM^{-} and B220^{+}IgM^{+} cells at day 3 of culture for the indicated genotypes.

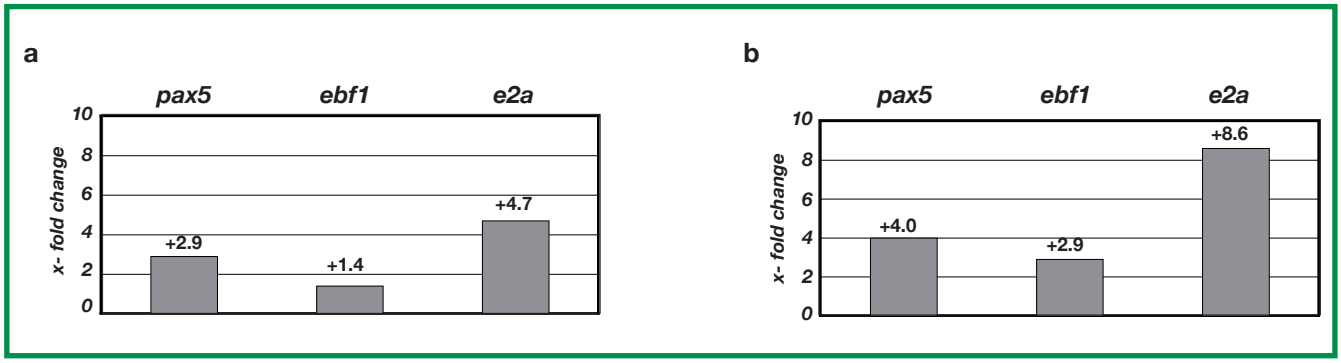

Fig. R16. Transcriptional levels of *pax5, ebf, e2a* and *c-myc* in pro- and pre-B cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* mice. B220^{+}IgM^{-} cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* mice, *c-myc$^{fl/fl}$; mx-cre^{-}* mice and *c-myc$^{fl/fl}$; mx-cre^{+}*mice were sorted; we then synthesised cDNA and used qRT-PCR to study the expression of Pax5, EBF and E2A transcription factors. (a) Comparison of transcriptional levels between cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* and *c-myc$^{fl/fl}$; mx-cre^{-}* mice. (b) Comparison of transcriptional levels between cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* and *c-myc$^{fl/fl}$; mx-cre^{+}*mice.

Comparison of *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* and *c-myc$^{fl/fl}$; mx-cre* mice showed upregulation of *pax5* and *e2a* transcriptional levels by 2.9- and 4.7-fold, respectively, whereas no substantial differences were found for *c-myc* and *ebf1*. When we compared the levels for these genes in B220^{+}IgM^{-} cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* and homozygous *c-myc$^{fl/fl}$; mx-cre^{+}* mice, we found upregulated transcription for *pax5*, *ebf1* and *e2a* (4.0-, 2.9-, and 8.6- fold, respectively) (Fig. R16a). These data suggest that ectopic Pax5 expression in pro- and pre-B cells from *Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}* is able to restore the transcriptional program of the E2A/EBF/Pax5 network of transcription factors.

Discussion

5. Discussion

In this study, we provide insight into the role of the proto-oncogene *c-myc* in B lymphopoiesis. Using conditional mouse models bred with distinct stage-specific *Cre* recombinase strains, we show an essential function for c-Myc in B cell development. We identified several genes that, together with c-Myc, might contribute to this developmental process. To test our hypothesis, we performed gain-of-function studies that demonstrate that c-Myc regulates the expression of key transcription factors involved in the specification and commitment of lymphoid precursors towards the B cell lineage.

The use of two mouse models that inactivate *c-myc* at different time points has allowed us to establish a new mechanism of c-Myc function in B cell development. In the first model, *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ inactivates *c-myc* in a constitutive fashion specifically in cells of the B lineage, allowing study of B lymphopoiesis in c-Myc-deficient immature B cells. In the second model, *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ (Baena et al., 2005), inactivation of *c-myc* is under the control of an inducible promoter (*mx1*). This results in *c-myc* deletion in several organs, with highest efficiency in liver and BM. These mice inactivate *c-myc* starting at the HSC stage, (Baena et al., 2007) and are therefore an appropriate tool with which to study B lymphopoiesis from c-Myc-deficient haematopoietic precursors.

To study B cell development in the BM of these mice, we analyzed a variety of cell surface markers that define immature B cell subsets. In the case of *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice, we found a blockade in the B cell developmental process and detected only cells of the B220^{+}IgM^{-} and B220^{+}CD43^{+} phenotype. The absolute numbers of B220^{+} and B220^{+}IgM^{-} cells were also reduced by two- and three-fold, respectively. In the case of *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$, there was a reduction in both the relative and absolute numbers of the immature B cell subpopulations defined by B220^{+}IgM^{-} and B220^{lo}IgM^{+}cells. The data generated in both mouse models suggest a requirement for the *c-myc* proto-oncogene in B cell development. In *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice, *c-myc* inactivation begins at the pro-B cell stage; pro-B cells are consequently c-Myc-deficient, but the pool of precursors that give rise to B cells are wild-type cells. In contrast, in *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice, *c-myc* is deleted from the earliest progenitors in the BM, i.e., BM precursor cells, and give rise to c-Myc-deficient B lymphocytes.

In *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$*; ROSA26-egfp* mouse spleen, we detected some B220^{hi}IgM^{+} cells, although many fewer than in wild-type mice (16.8-fold decrease). To determine whether the block in the B cell differentiation process of c-Myc-deficient cells was due entirely to *c-myc* inactivation, we cultured IgM-depleted B lymphocytes *in vitro* with appropriate cytokines to allow B lymphocyte expansion. Our findings demonstrate that c-Myc-deficient BM cells cannot give raise to IgM-expressing mature B cells, either *in vivo* or *in vitro*. These experiments clearly demonstrate that the B cell developmental blockade is an intrinsic failure of c-Myc-deficient immature B lymphocytes, and that the mature B cells found in the spleens of these mice are not generated in the absence of c-Myc. These data concur with our previous finding that pro-, pre- and immature B cells die by apoptosis due to *c-myc* inactivation.

The process of B cell differentiation comprises a series of proliferation, apoptosis and differentiation steps from early committed precursors to mature B cells; these steps are regulated by lineage-specific and non-specific genes. Of the various biological *in vivo* functions of c-Myc, induction of apoptosis and cell

proliferation are currently the best characterised. As several studies have correlated c-Myc expression with apoptosis in various cell lines, we analysed apoptosis in c-Myc-deficient B lymphocytes. We obtained similar results in *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ and *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice. c-Myc-deficient immature B lymphocytes (pro- and pre-B) from *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice showed increased apoptosis compared to wild-type cells. In *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice, we observed an increase in the relative numbers of apoptotic B220^{lo}IgM^{-} and B220^{lo}IgM^{+} cells in the BM of homozygous mice compared to wild-type control cells. These results suggest that when cells inactivate *c-myc*, they die by apoptosis. Attempts to rescue the survival of c-Myc-deficient B lymphocytes were previously tried in our lab by breeding *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice with Bcl-2 transgenic mice, which overexpress the potent Bcl-2 anti-apoptotic protein. The resulting mice express Bcl-2 in c-Myc-deficient BM cells. However, experiments carried out with these mice showed that Bcl-2 did not inhibit cell death of B lymphocytes. When we studied proliferation of immature B lymphocytes, we found that immature B cells (pro- and pre-B) were able to proliferate in the absence of c-Myc, although at a lower rate that wild-type cells. This led to the hypothesis that at this developmental stage, B cell proliferation is a c-Myc-independent event or that other genes, of the Myc family or for other transcription factors, compensate the c-Myc defect. These data suggest that *c-myc* is not essential for pro- and pre-B cell proliferation, but could compromise optimal proliferation of these cells.

Gene expression analysis in c-Myc-deficient pro- and pre-B cells showed that the levels of several transcription factors known to be key regulators of B lineage specification and commitment were downregulated in these cells. Transcription levels of *tcfe2a* (which codes for the E2A protein) and *ebf1* (which encodes EBF) were downregulated in c-Myc-deficient cells by 6.9- and 2.3-fold, respectively, compared to wild-type cells. These transcription factors are important regulators at the earliest stages of B cell differentiation, from common lymphoid progenitors to pro-B cells (Bartholdy and Matthias, 2004; Medina et al., 2004). Moreover, *pax5* transcription levels were downregulated by 17-fold in c-Myc-deficient B220^{+}IgM^{-} cells. The Pax5 transcription factor plays an important role in commitment and the maintenance of B cell identity (Fuxa and Skok, 2007). In addition, Pax5 regulates the expression of B lineage-specific genes such as *blnk*, *N-myc* and *cd79a*, which were also downregulated in c-Myc-deficient pro- and pre-B cells. Our findings suggest that the differentiation block caused by *c-myc* inactivation is caused by the regulation of potential downstream Myc targets such as E2A, EBF and Pax5.

Knock-out mouse models have been generated for E2A, EBF and Pax5, and have shown that absence of these proteins leads to blockages in B cell differentiation, either at the onset of B lineage commitment or at the beginning of V_H-to-DJ_H recombination. E2A- and EBF-deficient mice show a developmental block at the pre-pro-B cell stage (or Fraction A) and fail to rearrange D_H-to-J_H segments (Bain et al., 1994; Lin and Grosschedl, 1995). The Pax5-deficient mice show a developmental block at the pro-B cell stage, with impaired $V_{H\ distal}$-to-DJ_H recombination (Nutt et al., 1997; Urbanek et al., 1994). We analysed the rearrangement status of the IgH chain in c-Myc-deficient pro- and pre-B cells and found impaired rearrangements for D_H-to-J_H, $V_{H\ proximal}$-to-DJ_H and $V_{H\ distal}$-to-DJ_H segments. *c-myc* inactivation in pro- and pre-B cells therefore causes defective Ig heavy chain rearrangement. We nonetheless cannot conclude whether this impairment is caused by the downregulated E2A, EBF and/or Pax5 transcription factor levels or that it is likely that the impairment in V(D)J recombination is a secondary effect.

CD19 expression is considered the hallmark of B cell commitment by B cell precursors, and is also a direct target gene of the Pax5 transcription factor. CD19 expression begins at the pro-B cell stage and its expression is maintained up to the mature B cell stage. We found greatly reduced CD19 expression levels in pro- and pre-B cells from c-Myc-deficient bone marrow. Analysis of cell surface CD19 levels Hardy fractions A, B, C and C' from wild-type and *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice confirmed a notable reduction in this

protein in mutant mouse fraction B, which corresponds to the onset of CD19 expression. Expression of pre-BCR acts as an important developmental checkpoint by controlling the pro- to pre-B cell transition. The pre-BCR is a complex formed by Igμ, the surrogate light chains λ5 and VpreB, and the signal-transducing proteins Igα and Igβ. Mice deficient in E2A, EBF or Pax5 fail to express the pre-BCR on the surface of large pre-B cells, due to downregulated expression of genes that encode one or more of the pre-BCR components. c-Myc-deficient B cells expressed lower pre-BCR levels on their surfaces. These findings further support the hypothesis that downregulation of EBF and Pax5 levels account for the B cell differentiation blockade in c-Myc-deficient B cells.

Another characteristic feature of Pax5-deficient B cells is that they are uncommitted progenitors that can give rise to other haematopoietic lineages, both *in vivo* and *in vitro* (Nutt et al., 1999; Rolink et al., 1999). We tested the *in vitro* plasticity of c-Myc deficient pro-B cells from *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice. These cells did not generate myeloid nor erythroid lineage cells; they generated only B lineage cells and less efficiently than wild-type cells. The few colonies obtained in the case of colony-forming erythrocytes and granulocytes/macrophages were considered to be background levels, as in similar assays wild-type BM progenitors normally give rise to larger numbers of colonies (25-150 colonies per dish). Our findings suggest that c-Myc-deficient pro-B cells are B lineage-committed cells that cannot progress to more mature B cell differentiation stages because they die due to *c-myc* inactivation.

To determine whether Pax5 downregulation cooperated with c-Myc in the impairment in B cell differentiation observed in c-Myc-deficient mice, we performed gain-of-function studies for Pax5. We used a conditional mouse model in which Pax5 is overexpressed in distinct cells of the haematopoietic lineage cells, depending on the breeding of *Ik*$^{neo/+}$ mice with mice bearing different stage-specific *Cre* recombinase genes. Two mouse models expressing Pax5 in c-Myc-deficient BM cells were generated, one with specific Pax5 expression in the pro-B cell subpopulation (*Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$) and the other in which expression began in early haematopoietic precursors (*Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^{+}$). Our attempts to rescue B cell development by generating *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ were unsuccessful. Cells were unable to progress beyond the B220^{+}IgM^{-} or B220^{+}CD43^{+} stages of differentiation, and also showed acute reduction in relative numbers of the B220^{+}CD19^{+} subpopulation. On the contrary, *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice showed an increase in both relative and absolute numbers of B220^{+}IgM^{-} and B220^{lo}IgM^{+} subpopulations compared to *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice (2.4- and 5-fold increase in absolute numbers, respectively). Relative numbers of the B220^{lo}CD43^{-} subpopulation (Hardy fraction E or small pre-B cells) also showed a substantial increase (3.1% vs. 0.8%, 3.9-fold increase) compared to the same population in *c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice. The differences between the two mouse models could be attributed to the timing of Pax5 expression and of *c-myc* inactivation. In the case of *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$mice, the inability to overcome c-Myc deficiency might be caused by the brief time window available for Pax5 expression before cells die due to *c-myc* deletion, whereas in *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice, Pax5 might be expressed before the cell inactivates *c-myc*, allowing the cell to proceed through the B cell differentiation program.

IgM-depleted BM cells were cultured and expanded *in vitro* in B lineage conditions to determine whether these conditions could rescue cells from the developmental block and generate IgM-positive cells *in vitro* in *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ and *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice. In the case of *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice, BM and pro-B cells generated neither IgM-positive cells nor B220^{+}CD19^{+} cells *in vitro*. Not only was the ability to generate IgM-positive cells reduced, but the capacity to produce B lineage cells was also diminished at culture day 3. *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ BM cells, thus showed no obvious differences relative to *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ cells. Surprisingly, Pax5-expressing c-Myc-deficient BM cells from *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice generated IgM-positive cells after 3 days in culture, while cells

from *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$ mice did not (9.3% vs. 0.6%; 15.5-fold increase); absolute numbers these cells were also increased in *Ik*$^{neo/+}$; *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$ mice. In addition, Pax5-expressing cells from *Ik*$^{neo/+}$; *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$ mice showed a restored capacity to generate B220^{+}CD19^{+} cells compared to c-Myc-deficient cells from *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$ mice (31.2% vs. 8.2%; 3.8-fold increase). Our experimental findings suggest that whereas Pax5 expression in c-Myc-deficient haematopoietic precursors is able to restore the capacity of these cells to progress through the B cell developmental pathway, it is insufficient to confer this advantage when expressed in c-Myc-deficient pro-B cells.

Our results suggest that c-Myc could be modulating cell fate by direct or indirect regulation of transcription factors involved in lineage cell commitment. Regarding B and T lymphocytes, c-Myc is expressed in both cell types (Douglas et al., 2001) and is necessary for the differentiation of both B and T lymphocytes. c-Myc-deficient T lymphocytes are blocked at the DN stage of development and fail to undergo further differentiation into DP cells (Douglas et al., 2001). It is not known at which exact developmental stage the T versus B cell lineage commitment takes place, but it is thought that low Notch1 activity is sufficient to inhibit B cell differentiation, whereas higher Notch1 activity might be sufficient to drive T cell differentiation (Schmitt et al., 2004; Tan et al., 2005). Overall, one possibility could be that c-Myc might reinforce the cell fate decision once the cell has already committed to either the B or T lineage.

Our results show that, when expressed by early precursors in the BM, Pax5 rescues B cell development by c-Myc-deficient lymphocytes. Pax5 and EBF are key transcription factors in B cell specification and commitment, and *Pax5* transcription is induced by *Ebf1* (Medina et al., 2004; O'Riordan and Grosschedl, 1999). Pax5 expression is needed to maintain commitment to the B lineage, which is achieved in part by the creation of a feedback loop that sustains EBF expression throughout the life of a B cell (Roessler et al., 2007). Experiments should be carried out to test EBF transcriptional levels in rescued Pax5-expressing c-Myc-deficient B cells. It remains to be determined whether EBF expression or restoration of E2A expression in c-Myc-deficient BM cells would be sufficient to rescue B cell differentiation by "switching on" the B cell developmental program. Based on our findings, we hypothesize a model in which c-Myc could regulate the E2A-EBF-Pax5 network of transcription factors, which have crucial roles at

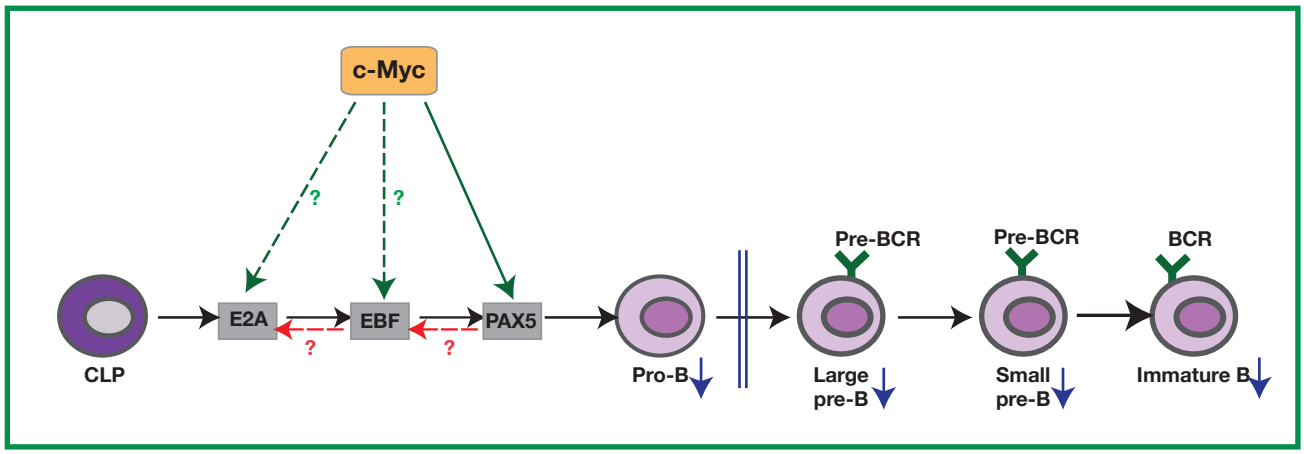

Figure D1. Proposed mechanism for the role of the proto-oncogene c-myc in the control of B cell development. Dashed lines represent open questions. The parallel blue lines represent the developmental block observed in *c-myc*$^{fl/fl}$; *mb1*$^{cre/+}$ mice, and blue arrows indicate the reduction in the numbers of the distinct B cell populations observed in *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$ mice.

the onset of B cell development. The mechanism by which c-Myc controls B cell differentiation could be clinically relevant for c-Myc-induced tumours such as Burkitt's lymphoma and leukaemia. Identification of Myc target genes is of extreme importance, given their potential as therapeutic targets in the treatment of these human cancers. To integrate these findings into a model (Fig. D1), we speculate that the *c-myc* proto-oncogene controls the B cell differentiation program by regulating the expression of transcription factors involved in B cell commitment (such as Pax5 and probably EBF), and in turn influencing the survival of pro-, pre- and immature B cells.

Conclusions

6. Conclusions

1. *c-myc* is necessary for the survival and maintenance of pro-B, pre-B and immature B lymphocytes.

2. Pro-B, pre-B and immature B lymphocytes are able to proliferate in the absence of *c-myc*.

3. Inactivation of *c-myc* in pro-B and pre-B cells results in impaired V(D)J recombination in *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice.

4. Gene expression levels of the E2A, EBF and Pax5 transcription factors are downregulated in c-Myc-deficient B220^{+}IgM^{-} cells from *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ mice.

5. c-Myc-deficient pro-B and pre-B lymphocytes have reduced cell surface expression levels of CD19 and pre-BCR.

6. Ectopic Pax5 expression from the *Ikaros* endogenous locus is able to promote B cell differentiation from B220^{+}IgM^{-} to B220^{lo}IgM^{+} cells *in vitro* in *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ mice.

7. Ectopic Pax5 expression in pro- and pre-B cells from *Ik*$^{neo/+}$*; c-myc*$^{fl/fl}$*; mx-cre*$^{+}$ is able to restore the transcriptional program of the E2A/EBF/Pax5 network of transcription factors.

Resumen

7. Resumen

7.1. Introducción

Las proteínas Myc (N-, L- y c-) son miembros de la familia de factores de transcripción "basic/helix-loop-helix/leucine zipper", implicadas en la regulación de la proliferación celular, tamaño celular, diferenciación y apoptosis (Askew et al., 1991; Evan et al., 1992; Facchini LM and LZ, 1998; Henriksson and Luscher, 1996; Kelly et al., 1983; Waters et al., 1991; Wisden et al., 1990). La expresión de *c-myc* es ubícua y está modulada a lo largo del ciclo celular. Así, la estimulación mitógenica de células quiescentes incrementa la expresión de *c-myc* a niveles máximos en la transición G0/G1 a S, disminuyendo progresivamente a lo largo del ciclo celular (Campisi et al., 1984; Kelly et al., 1983). La desregulación de la expresión de *c-myc* está implicada en el desarrollo de distintas patologías, tanto en ratón como en humanos, tal como linfoma de Burkitt, cáncer de mama o de pulmón (Adams and Cory, 1985; Dalla-Favera et al., 1982; Leder et al., 1986; Morgenbesser and DePinho, 1994; Spanopoulou E et al., 1989). Sólo en Estados Unidos, se estima que la frecuencia de alteraciones genéticas de *c-myc,* en cánceres humanos, causa del orden de 100.000 muertes al año.

Las evidencias experimentales que implican a *c-myc* en procesos proliferativos y apoptóticos están basadas, abrumadoramente, en líneas celulares. La expresión ectópica de *c-myc* en células quiescentes induce la entrada en la fase S del ciclo celular y acorta su duración en diferentes tipos celulares (Eilers et al., 1991; Palmieri S et al., 1983; Stern DF, 1986). Tanto su capacidad para promover la entrada en el ciclo celular, como su regulación durante el mismo, llevaron a pensar que c-Myc podría estar modulando genes responsables del control del ciclo. Así, multitud de datos experimentales indican que *c-myc* induce la proliferación celular activando genes reguladores positivos del ciclo celular (Mateyak et al., 1997; Zornig and Evan, 1996) como de *ciclina d1, d2 , e, a* y *cdc25A*, o reprimiendo inhibidores del ciclo celular como *p21* y *p27* (Bouchard et al., 1999; Daksis JI, 1994; Galaktionov et al., 1996; Hoang AT et al., 1994). Otra de las funciones más estudiadas de c-Myc es la apoptosis. La primera prueba de la implicación de c-Myc en apoptosis, se observó en fibroblastos cuando la sobreexpresión de *c-myc*, en condiciones limitantes de suero, provocaba muerte celular programada de los mismos (Askew et al., 1991; Evan et al., 1992; (Thompson et al., 1998). A raíz de estos resultados, surgieron multitud de estudios que correlacionaban una modulación positiva o negativa de la expresión de *c-myc* con apoptosis en diferentes tipos celulares.
En el caso de linfomas B humanos y murinos, la apoptosis inducida a través del receptor de la células B (BCR) correlaciona con una modulación negativa de la expresión de *c-myc* (Brunner T et al., 1995; Dhein J et al., 1995; Ju ST et al., 1995). En contraste con estos resultados, se ha demostrado que linfocitos primarios B deficientes en c-Myc tienen una mayor resistencia a la muerte inducida a través de CD95-CD95L o a la muerte espontánea en cultivo (de Alboran et al., 2004). En líneas generales, todos estos trabajos parecen indicar que la función de c-Myc en apoptosis, proliferación o diferenciación no sólo depende del tipo celular sino del estadío de diferenciación en el cual se encuentra la célula.

c-Myc se expresa durante todos los estadíos de diferenciación de los linfocitos B (Morrow et al., 1992; Zimmerman and Alt, 1990) . Los linfocitos B son generados a partir de los progenitores pluripotentes por un proceso altamente controlado que tiene lugar en el hígado fetal, y en la médula ósea y los órganos linfoides secundarios, en la fase adulta . El proceso

de desarrollo de los linfocitos B, está íntimamente ligado al reordenamiento de las cadenas pesada y ligera de las inmunoglobulinas. El primer estadío de diferenciación, se conoce como pre-pro-B (o fracción A), en el cual ambas cadenas se encuentran en la configuración germinal. En la siguiente fase de desarrollo, conocida como pro-B (compuesta por las fracciones B y C), se inicia el reordenamiento de la cadena pesada, que tiene lugar de forma secuencial, así, primero reordenan los segmentos D_H a J_H, seguidos por el reordenamiento de los segmentos V a DJ_H. Una vez completado el reordenamiento de la cadena pesada, la célula pasa al siguiente estadío de diferenciación pre-B (compuesto por las fracciones C' y D) en el que empieza el reordenamiento de la cadena ligera, V a J_L. Cuando ambas cadenas han reordenado, la célula se convierte en una célula inmadura B (o fracción E), caracterizada por la expresión de IgM en su superficie. Todas estas fases de diferenciación tienen lugar en la médula ósea (MO), pero las células inmaduras B migran a la periferia, donde están sujetas a un proceso de selección tras el cual las células supervivientes se convierten en células maduras B, caracterizadas por la expresión de los marcadores IgM e IgD en su superficie. Los estadíos de diferenciación de los linfocitos B, están caracterizados por la expresión en membrana de distintos marcadores celulares como son B220, CD43, CD19, IgM e IgD.

La generación de linfocitos maduros B requiere una serie de decisiones de destino celular en las cuales se han descrito varios factores de transcripción que juegan un papel relevante. Entre todos ellos, por lo menos cinco han demostrado ser esenciales para el desarrollo de los linfocitos B. Éstos son: PU.1, Ikaros, E2A, EBF (early cell factor) , Pax 5 (paired domain 5). PU.1 e Ikaros son necesarios para la formación de los progenitores linfoides (Medina et al., 2004), E2A y EBF son importantes en los estadíos tempranos de diferenciación de los linfocitos B (Medina et al., 2004) y el factor de transcripción Pax-5 desempeña un papel crítico en el compromiso hacia linaje B y diferenciación de los linfocitos B (Nutt et al., 1999) . Pax-5 regula la diferenciación de las células B activando genes que confieren identidad B como son *cd19* (Kozmik et al., 1992), *blnk* (Schebesta et al., 2002), *cd79a* (Fitzsimmons et al., 1996) y reprimiendo genes de linaje T como *notch-1* (Souabni et al., 2002). Algunos tumores presentan una desregulación de la expresión de Pax-5. En este sentido, Pax-5 se encuentra sobreexpresado en varios linfomas Non-Hodgkin que presentan translocaciones que implican a los loci de Pax-5 y IgH (Souabni et al., 2007; Zhang et al., 2003) .

La función del proto-oncogén *c-myc* en la diferenciación de los linfocitos B ha sido poco estudiada. De todos los miembros de la familia Myc, sólo c- y N-Myc se expresan en los linfocitos B. *c-myc* se expresa a diferentes niveles durante el desarrollo de los linfocitos B: su expresión empieza en el estadío por-B y es inducida en respuesta a citoquinas, como IL-7 (Morrow et al., 1992), se expresa junto con N-myc en las células pre-B, y en células B inmaduras y maduras tras su activación. Estudios en líneas celulares de linfocitos, han demostrado que la desregulación de c-Myc induce la muerte celular por apoptosis (Thompson, 1998). Estudios *in vivo*, han demostrado que la sobreexpresión de c-Myc en linfocitos B, resulta en una linfopoiesis B anómala caracterizada por un incremento en el número de linfocitos pro- y pre-B y una reducción de las poblaciones B inmaduras y maduras de la médula ósea y en una reducción de los linfocitos B del bazo (Iritani and Eisenman, 1999). Asimismo, estos linfocitos tienen un tamaño mayor y este aumento está en relación con un incremento en la síntesis de proteínas.

El campo de c-Myc ha carecido, hasta hace unos años de modelos in vivo basados en pérdida de función, debido a la letalidad embrionaria (día 9-10) del KO en línea germinal de ratón (Davis et al., 1993) . La generación de un modelo condicional de ratón (de Alboran et al., 2001) que permite la inactivación funcional de *c-myc* mediante la utilización del sistema cre-lox (Gu et al., 1993; Thomas and Capecchi, 1987) ha sido de gran ayuda para estudiar su función *in vivo* en individuos adultos. Estudios realizados en linfocitos B primarios deficientes en c-Myc, han revelado que el proto-oncogén *c-myc* juega un papel determinante

en la fisiología de los linfocitos B maduros. c-Myc regula la proliferación celular a través del inhibidor de ciclo celular P27 (de Alboran et al., 2001) y participa, de forma sorprendente, tanto en la muerte celular programada como en la muerte espontánea en linfocitos maduros (de Alboran et al., 2003).

7.2. Resultados

7.2.1. Generación de modelos animales para el estudio de la función de c-Myc en el desarrollo de los linfocitos B

Para llevar a cabo el estudio de la función de c-Myc en el desarrollo de los linfocitos B generamos dos modelos animales de ratón en los que la inactivación de este proto-oncogén tiene lugar de forma constitutiva o inducible.

7.2.1.1. Modelo constitutivo: *c--myc$^{fl/fl}$;- mb--1$^{cre/+}$*

Los ratones *c--myc$^{fl/fl}$* se cruzaron con los ratones *mb--1$^{cre/+}$* (Hobeika et al., 2006), que expresan la recombinasa *cre* bajo el promotor del gen *mb--1* el cual codifica la subunidad Igα□ del pre-receptor de los linfocitos B (pre-BCR). Estos ratones inactivan *c--myc* de forma constitutiva y específicamente en las células pro-B. Para poder seleccionar las células deficientes en c--Myc por la expresión de green fluorescent protein (GFP), los ratones *c--myc$^{fl/fl}$;- mb--1$^{cre/+}$* se cruzaron con los ratones *Rosa26-egfp* (Mao et al., 2001), que expresan la proteína GFP bajo el promotor endógeno del gen *rosa26*.

7.2.1.2. Modelo inducible: *c-myc$^{fl/fl}$;-mx-cre^{+}*

Los ratones *c-myc$^{fl/fl}$* se cruzaron con los ratones transgénicos para *cre* bajo el promotor inducible por interferon *mx*. La inoculación de interferon alfa o el ácido polyinosinic-polycytidylic (pIpC), permite inducir la inactivación de *c-myc* en médula ósea (MO) e hígado (Kuhn et al., 1995).

7.2.2. c-Myc es necesario para el desarrollo de los linfocitos B

Durante el proceso de diferenciación de linfocitos B se produce el reordenamiento de los genes del receptor de las células B (BCR). Este reordenamiento de los genes del BCR define los estadíos de diferenciación de linfocitos B, pro- y pre-B ($B220^{+}IgM^{-}$), inmaduros ($B220^{lo}IgM^{+}$) y células B maduras ($B220^{hi}IgM^{+}$). La inactivación de c-Myc en linfocitos B inmaduros en la MO de los ratones *c-myc$^{fl/fl}$;-mb-1$^{cre/+}$*provoca un bloqueo en la diferenciación de linfocitos B en el estadío $B220^{+}IgM^{-}$. La caracterización de los linfocitos B presentes en el bazo de los ratones *c-myc$^{fl/fl}$;-mb-1$^{cre/+}$*, mostró que tanto los números relativos y absolutos mostraban una reducción de la población de células $B220^{+}IgM^{+}$ en comparación con los ratones control.

Por otro lado, los ratones *c-myc$^{fl/fl}$;-mx-cre^{+}* mostraron una diferenciación anómala de los linfocitos B caracterizada por una disminución de las poblaciones $B220^{+}IgM^{-}$, y $B220^{lo}IgM^{+}$.

La MO de ratones *c-myc$^{fl/fl}$;-mb-1$^{cre/+}$*fue deplecionada de las células IgM positivas y puesta en cultivo en condiciones que favorecen la expansión de linfocitos B *in vitro*. Estos cultivos no generaron células IgM^{+}, indicando que las células $B220^{+}IgM^{+}$ presentes en el bazo de los ratones *c-myc$^{fl/fl}$;-mb-1$^{cre/+}$* no podían ser generadas en ausencia de c-Myc.

7.2.3. c-Myc es necesario para la supervivencia de los linfocitos pro-, pre- y los linfocitos B inmaduros

La inactivación de c-Myc en la MO de los ratones *c-myc$^{fl/fl}$;-mb-1$^{cre/+}$* y *c-myc$^{fl/fl}$;-mx-cre^{+}* produce un aumento en los niveles de apoptosis de los linfocitos $B220^{+}IgM^{-}$ en el caso de los ratones *c-myc$^{fl/fl}$;-mb-1$^{cre/+}$*, y de los linfocitos $B220^{+}IgM^{-}$ y $B220^{lo}IgM^{+}$ en ratones *c-myc$^{fl/fl}$;-mx-cre^{+}*.

7.2.4. Los linfocitos B son capaces de proliferar en ausencia de c-Myc

Ensayos de incorporación de BrdU en los linfocitos B, mostraron que las células B220^{+}IgM^{-} de ratones *c-myc$^{fl/fl}$;-mb-1$^{cre/+}$*, y los linfocitos B220^{+}IgM^{-} y B220^{lo}IgM^{+} de ratones *c-myc$^{fl/fl}$;-mx-cre^{+}* proliferan en ausencia de c-Myc, aunque menos que las mismas células de ratones control.

7.2.5. Patrón de expresión génica en linfocitos B inmaduros y deficientes en c-Myc

Utilizando "real time" PCR observamos que las células B deficientes en c-Myc presentaban niveles de expresión reducidos de *e2a, ebf1 y pax5*. En el caso de Pax5, no sólo sus niveles de expresión estaban disminuidos sino que también los de sus genes diana como son *blnk, n-myc, Ig*α e *Ig*β.

7.2.6. Los linfocitos B inmaduros deficientes en c-Myc presentan una recombinación V(D)J anómala

Mediante PCR semicuantitativa y el uso de oligonucleótidos específicos para la detección de segmentos reordenados D_H-$J_{H,}$ $V_{Hproximal}$-DJ_H and $V_{Hdistal}$-DJ_H, estudiamos la recombinación V(D)J en células B220^{+}IgM^{-} sorteadas de ratones *c-myc$^{fl/fl}$;-mb-1$^{cre/+}$* y ratones control. Los resultados mostraron que los linfocitos pro- y pre-B deficientes en c-Myc presentaban una recombinación V(D)J anómala.

7.2.7. La células pro-y pre-B deficientes en c-Myc tienen una expresión en membrana de CD19 y pre-BCR reducida

El estudio de la expresión en membrana de los marcadores CD19 y pre-BCR, mediante citometría de flujo, mostró que las células pro- y pre-B deficientes en c-Myc, presentan una expresión reducida de dichos marcadores en relación a su expresión en las células de ratones control.

7.2.8. Los linfocitos B deficientes en c-Myc no son capaces de generar células de otros linajes hematopoyéticos *in vitro*

Se llevaron a cabo ensayos clonogénicos para estudiar la capacidad de los linfocitos pro- y pre-B de los ratones *c-myc$^{fl/fl}$;-mb-1$^{cre/+}$* de generar células de linaje eritroide, granulocítico/mieloide y linfoide. Los resultados mostraron que los linfocitos pro-B deficientes en c-Myc sólo fueron capaces de generar células de linaje B, indicando que son células comprometidas a ese linaje.

7.3. Expresión ectópica de Pax5 en linfocitos B deficientes en c-Myc

Con el fin de estudiar si la expresión ectópica de Pax5 en células deficientes en c-Myc era suficiente para rescatar la diferenciación anómala de los linfocitos B, cruzamos los ratones *Ik$^{neo/+}$* (que expresan el gen *pax5* bajo el promotor de *ikaros* endógeno) con las cepas de ratón mencionadas anteriormente: *c-myc$^{fl/fl}$;-mb-1$^{cre/+}$* y *c-myc$^{fl/fl}$;-mx-cre^{+}*.

7.3.1. Los ratones *Ik$^{neo/+}$; c-myc$^{fl/fl}$;-mb-1$^{cre/+}$* presentan diferenciación anómala de los linfocitos B

La caracterización por citometría de flujo de las distintas poblaciones de linfocitos B presentes en la MO de los ratones *Ik$^{neo/+}$; c-myc$^{fl/fl}$;-mb-1$^{cre/+}$*, mostró que estos linfocitos se encontraban bloqueados en el estadío de diferenciación B220^{+}IgM^{-}. Asimismo, la expresión en superficie del marcador CD19 en estas células, estaba disminuida en los ratones *Ik$^{neo/+}$; c-myc$^{fl/fl}$;-mb-1$^{cre/+}$* en comparación con la expresión en las mismas células de los ratones control.

Células de la MO de los ratones *Ik$^{neo/+}$; c-myc$^{fl/fl}$;-mb-1$^{cre/+}$* , fueron deplecionadas de IgM, y se cultivaron en condiciones que favorecen la expansión de los linfocitos B. Los resultados mostraron que dichas células fueron incapaces de generar células B220^{+}IgM^{+}. Los resultados muestran que los ratones *Ik$^{neo/+}$; c-myc$^{fl/fl}$;-mb-1$^{cre/+}$* presentan un bloqueo en la diferenciación de los linfocitos B.

Mediante RT-PCR cuantitativa, analizamos la expresión génica de *e2a, ebf1* y *pax5* en estas células. Los resultados obtenidos demostraron que estas células

no llegan a expresar *pax5*, lo que podría explicar el bloqueo en la diferenciación de los linfocitos B en los ratones *Ik*$^{neo/+}$; *c-myc*$^{fl/fl}$;*-mb-1*$^{cre/+}$.

7.3.2. Rescate de la diferenciación de los linfocitos B *in vivo* en los ratones *Ik*$^{neo/+}$; *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$

En la MO de los ratones *Ik*$^{neo/+}$; *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$, inoculados previamente con pIpC, observamos un aumento, tanto en números absolutos como relativos, de las poblaciones B220^{+}IgM^{-} y B220^{lo}IgM^{+}, en comparación con la MO de los ratones *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$. Asismismo, también observamos un rescate en la expresión en membrana del marcador CD19 en las células B220^{+}IgM^{-} de ratones *Ik*$^{neo/+}$; *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$.

El cultivo de células de la MO de los ratones *Ik*$^{neo/+}$; *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$, previamente deplecionadas de IgM, en condiciones favorecedoras de la expansión de los linfocitos B, generó células B220^{+}IgM^{+}. Los resultados muestran que los ratones *Ik*$^{neo/+}$; *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$ presentan un rescate en el bloqueo de la diferenciación de los linfocitos B.

El estudio de la expresión génica de *e2a, ebf1* y *pax5* en células B220^{+}IgM^{-} de ratones *Ik*$^{neo/+}$; *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$, mostró que los niveles de expresión de estos genes estaban modulados positivamente en comparación con las mismas células de los ratones *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$.

7.4. Conclusiones

1. *c-myc* es necesario para la supervivencia y el mantenimiento de los linfocitos pro-B, pre-B y B inmaduros.

2. Los linfocitos pro-B, pre-B y B inmaduros son capaces de proliferar en ausencia de *c-myc*.

3. La inactivación de *c-myc* en los linfocitos pro-B y pre-B de los ratones *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$ provoca una recombinación V(D)J anómala

4. Los niveles de expresión génica de los factores de transcripción E2A, EBF and Pax5 se encuentran disminuidos en las células B220^{+}IgM^{-} de los ratones *c-myc*$^{fl/fl}$*; mb-1*$^{cre/+}$.

5. Los niveles de expresión de los marcadores de membrana CD19 y pre-BCR están reducidos en los linfocitos pro-B y pre-B deficientes en c-Myc.

6. La expresión ectópica de Pax5 controlada por el promotor del gen *Ikaros* endógeno, promueve la diferenciación de los linfocitos B del estadío B220^{+}IgM^{-} al B220^{lo}IgM^{+} *in vitro* en los ratones Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+}.

7. La expresión ectópica de Pax5 en los ratones Ik$^{neo/+}$; c-myc$^{fl/fl}$; mx-cre^{+} rescata el programa transcripcional formado por los factores de transcripción E2A/EBF/Pax5.

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Appendix

c-Myc regulates cell size and ploidy but is not essential for postnatal proliferation in liver

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The c-Myc protein is a transcription factor implicated in the regulation of multiple biological processes, including cell proliferation, cell growth, and apoptosis. *In vivo* overexpression of *c-myc* is linked to tumor development in a number of mouse models. Here, we show that perinatal inactivation of c-Myc in liver causes disorganized organ architecture, decreased hepatocyte size, and cell ploidy. Furthermore, c-Myc appears to have distinct roles in proliferation in liver. Thus, postnatal hepatocyte proliferation does not require c-Myc, whereas it is necessary for liver regeneration in adult mice. These results show novel physiological functions of *c-myc* in liver development and hepatocyte proliferation and growth.

The correct balance between cell proliferation and cell death is essential for the development of multicellular organisms. The Myc proteins are basic region/helix–loop–helix/leucine zipper transcription factors involved in the regulation of cellular proliferation, apoptosis, and cell growth (1, 2). The Myc family includes three closely related genes, c-, N-, L-Myc, which share similar biological activities, and all three have oncogenic potential. On this regard, L-Myc and N-Myc have been shown to be functionally equivalent to c-Myc-dependent activities in c-Myc nullizygous fibroblasts (3). Deregulated expression of *c-myc* is usually associated with the development of tumors in mice and humans (4, 5). C-Myc expression promotes the transition from G_0/G_1 to S phase of the cell cycle in multiple cell types, including hepatocytes, by regulating cyclin/cyclin-dependent kinase complexes (6–8). The G_0/G_1 to S-phase transition observed in hepatocytes of regenerating livers after partial hepatectomy (PH) correlates with rapid induction of *c-myc* and *n-myc* transcripts (9, 10). In limiting serum conditions, *c-myc* overexpression induces apoptosis in fibroblast and myeloid cell lines (11, 12). In hepatocyte cell lines, c-Myc expression causes apoptosis by inducing oxidative stress, and inhibition of c-Myc expression sensitizes hepatocytes to TNF-induced apoptosis (13, 14).

Transgenic mice overexpressing *c-myc* in liver show increased proliferation and apoptosis and develop tumors after a long latency period (15). More recently, *c-myc* has been shown to play an important role in the regulation of cell size in mice and flies (16, 17). In liver, hepatocytes can augment cell size by increasing cell ploidy through an altered cell cycle without cytokinesis (18). Interestingly, *c-myc* has been shown to regulate cell ploidy in hepatocytes from transgenic mice (19). Whether c-Myc regulates hepatocyte size is not clear. According to Trumpp *et al.* (20), c-Myc controls cell number instead of cell size in the organs of mice expressing hypomorphic alleles of *c-myc*. In this regard, a new role for *Drosophila* Myc has emerged in control of organ size by cell competition in flies (21, 22).

Germ-line inactivation of *c-myc* leads to multiple abnormalities and death at day 9–10 of embryonic development (23). Here, we used an inducible conditional approach to analyze the multiple functions of c-Myc in the context of liver growth in newborn and adult mice.

Materials and Methods

Mice. *c-myc*$^{fl/fl}$ conditional knockout mice (24) were bred with *mx-cre* mice (25), and progeny were bred to yield homozygous (*c-myc*$^{fl/fl}$;*mx-cre*$^{+}$) mice and control mice (*c-myc*$^{fl/+}$;*mx-cre*$^{+}$ and *c-myc*$^{fl/fl}$;*mx-cre*$^{-}$). Mice were genotyped by using a PCR-based analysis of tail genomic DNA described in ref. 26. To amplify the *mx-cre* transgene (PCR product, 269 bp), primers SF-4 (5′-GCATAACCAGTGAAACAGCATT GCTG-3′) and 69R (5′-GGACATGTTCAGGGATCGCCAGGCG-3′) were used.

Polyinosinic-Polycytidylic Acid (pIpC) Injections. *C-myc* deletion was induced in newborns (day 2 after birth) by four i.p. injections (300 μg each) of pIpC (Sigma) at 2-day intervals. For *in vitro* hepatocytes cultures, newborn mice received four injections in consecutive days. For liver regeneration experiments, 6-week-old mice received four i.p. injections of pIpC (500 μg each) at 2-day intervals.

Isolation and Culture of Mouse Hepatocytes. Hepatocytes from 6- to 7-day-old mice were isolated by collagenase A treatment (0.4 mg/ml, Roche Diagnostics, Mannheim, Germany) and culture in poly-DL-lysine-coated plates (1 mg/ml, Sigma) in M199 medium (10% FCS/2.20% $NaHCO_3$/1 mM glutamine/120 μg/ml penicillin/100 μg/ml streptomycin), EGF (20 ng/ml, GIBCO), and insulin (10 nM, Sigma) for 24 h at 37°C in an atmosphere of 7.5% CO_2 (27). Hepatocytes were cultured alone or with L-ascorbic acid (1 mM, Sigma). In older mice (6–10 weeks), hepatocytes were isolated following perfusion with collagenase A. For cell-cycle analysis, treated cells were permeabilized and propidium iodide-stained (DNA-Prep reagent kit, Beckman Coulter) and incubated (30 min at 37°C) before FACS analysis.

Histology and Immunohistochemical Staining. All organs were paraffin-embedded and H&E-stained (4-μm sections). Staining for TUNEL assays followed manufacturer's protocols (TMRred *in situ* cell death kit, Roche Diagnostics). Samples were counterstained with DAPI (Vector Laboratories). For proliferating cell nuclear antigen (PCNA) staining, we used a biotinylated anti-human PCNA antibody (Pharmingen). Biotinylated antibodies were developed by using a StrepABComplex/horseradish

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Abbreviations: pIpC, polyinosinic-polycytidylic acid; PCNA, proliferating cell nuclear antigen; DCFH-DA, dichlorodihidrofluorescin diacetate; RPA, ribonuclease protection assay; PH, partial hepatectomy; H&E, hematoxylin/eosin.

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peroxidase kit. Horseradish peroxidase was visualized with diaminobenzidine (Sigma).

***In Vivo* BrdUrd Labeling.** The thymidine analogue BrdUrd (1 mg/ml, Sigma), prepared fresh every 3 days, was administered in drinking water for 1 week. BrdUrd staining was performed by using anti-BrdUrd antibody (Becton Dickinson) following the manufacturer's protocol.

Dichlorodihidrofluorescin Diacetate (DCFH-DA) Staining. Freshly isolated hepatocytes were incubated (45 min at 37°C) with the oxidative-sensitive probe DCFH-DA (5 μM, Molecular Probes).

PH. *c-myc* deletion was induced by using pIpC in adult mice (6 weeks old). At 48 h after PH (28), liver was analyzed by PCNA Western blotting.

Single-Cell PCR for *flox* and *null* Alleles. Single PCNA-positive hepatocytes were isolated from paraffin-embedded sections by using a laser dissection technique (UV microdissection). Genomic DNA from a single hepatocyte was obtained by 1 h incubation at 56°C in 20 μl of lysis buffer (1× PCR buffer/0.5 mg/ml proteinase K/9 μg/ml tRNA). Proteinase K was inactivated by heating (at 95°C for 10 min), and nested PCR was performed to amplify *flox* or *null* alleles independently. First-round PCR was carried out with half of the lysate (10 μl) and primers Myc-fl-1 (TGATATCGAATTCCTGCAGCC) and Myc-fl-3′ (TTTTCTTTCCGATTGCTGAC) to amplify *flox* alleles. Remaining lysate (10 μl) was used for first-round PCR to amplify *null* alleles with primers Myc-Δ-S (TCGCGCCCCTGAATTGCTAGGAA) and Myc-Δ-5.1 (TCCAGGATGCTAGAGACCTTCTCT). Second-round PCR was performed with 5 μl of the first-round reaction mixture an internal third primer, Myc-fl-2.1 (GTGTCAAATAATAAGAGACACCTCCCT) for *flox* alleles (fl-1 + fl-2.1, 100 bp), and Myc-Δ-2.1 (TTTAGGACATTTAGGTCGAGGGAC) for *null* alleles (Δ-S + Δ-2.1, 180 bp). PCR conditions were 94°C 5 min, followed by 40 cycles (94°C for 30 sec, 60°C 30 for sec, and 72°C for 30 sec) at 72°C for 7 min.

Ribonuclease Protection Assay (RPA). Total RNA was isolated from liver with the RNEasy kit (Qiagen, Hilden, Germany). The multiprobe Rnase protection assay kit was used (Becton Dickinson). Riboprobes of Myc family genes (BD Riboquant multiprobe template sets, BD Pharmingen, San Diego) were radiolabeled [$^{32}\alpha$]UTP using an *in vitro* transcription kit (BD Riboquant, BD Biosciences, San Diego) according to manufacturer's protocol. Total RNA (10 μg) was hybridized with radiolabeled probes. RNase treatment and gel resolution of protected probes were according to manufacturer's protocol. The gel was developed on Kodak X-AR film with intensifying screen at −80°C.

Results and Discussion

To circumvent the lethality of *c-myc* knockout mouse and inactivate *c-myc* gene in liver, we bred *c-myc*$^{fl/fl}$ mice (24) with inducible *mx-cre* transgenic mice (23, 25). F_1 generation intercrosses originated homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ and littermate control mice that express Cre recombinase in multiple tissues after induction of the *mx* promoter with IFN-γ or pIpC (25). Non-pIpC-injected *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ newborn and adult mice showed no evident phenotype compared to control littermates (data not shown).

To delete the *c-myc* gene, we injected pIpC i.p. into newborn mice, four times in the first 8 days after birth (see *Materials and Methods*). PIpC-injected *c-myc*$^{fl/+}$;*mx-cre*$^{+}$ mice, *c-myc*$^{fl/fl}$;*mx-cre*$^{-}$ mice, and wt control mice had no apparent phenotype difference (data not shown). PIpC-injected 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice showed reduced body weight compared with controls (Fig. 1*F*). After perinatal pIpC injection, 10-day-old and

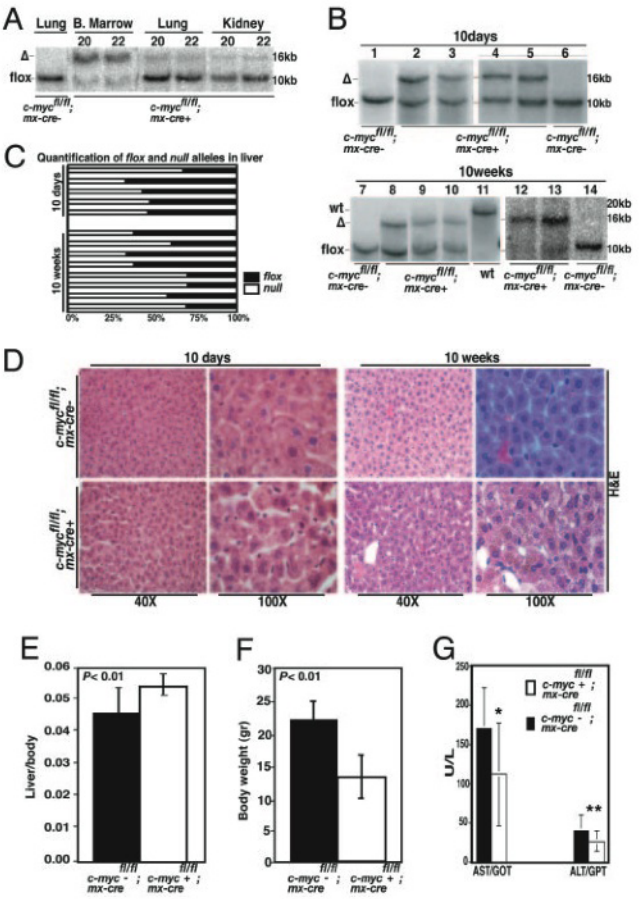

Fig. 1. Altered liver organization in homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. (*A*) Deletion of *c-myc* gene in homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Genomic DNA from the organs shown was isolated, *Eco*RI-digested, and probed as described (24). Two perinatally pIpC-injected 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice are shown. (*B*) Deletion of *c-myc* gene in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse liver. Genomic DNA from pIpC-injected 10 day-old and 10 week-old *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice was used. Southern blot was performed as in *A*. Numbers indicate individual mice. Genomic DNA from *c-myc*$^{fl/fl}$;*mx-cre*$^{-}$ mice or wt mice was used as a control. Experiment representative of multiple experiments. (*C*) Quantification of *flox* and *null* alleles in livers from pIpC-injected 10 day-old and 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Quantification was performed with PhosphorImager, using IMAGEQUANT software on Southern blots performed as in *B*. %A allele, A allele counts × 100/total alleles counts (flox plus null counts). *A* is either *null* or *flox* alleles. (*D*) H&E-stained sections from 10 day-old and 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ liver and control mouse liver. Seven 10-day-old mice of each genotype and seven 10-week-old mice of each genotype were analyzed. (*E*) Liver:body weight ratio of 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ and control mice. (*F*) Body weight of 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice from *E* (n = 30 for each genotype). (*G*) Hepatic function in 10-week-old mice. Serum levels of aspartate (AST/GOT) and alanine (ALT/GPT) aminotransferases. n = 14 for homozygous mice and n = 6 for control mice. U/L, units per liter. *, P = 0.0202; **, P = 0.0327 (Student's t test). In all experiments, mice were injected with pIpC as newborns (see *Materials and Methods*).

10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice showed minimal *c-myc* deletion (<10%) in kidney and lung (Fig. 1*A* and data not shown). Histology sections from these organs did not reveal any significant difference between homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control littermates (Fig. 5, which is published as supporting information on the PNAS web site). In contrast, efficient deletion of *c-myc* in bone marrow (≅90–100%) caused severe anemia in adult homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and death after 3 months

(Fig. 1*A*, data not shown, and E.B. and I.M.d.A., unpublished work).

After perinatal pIpC injection, livers from young (10 days old) and adult (10 weeks old) homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice showed efficient *c-myc* gene deletion (Fig. 1 *B* and *C*). The extent of *c-myc* deletion in liver (≅40–75%) was different among homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice (Fig. 1 *B* and *C*). These values were underestimated because of the differential transfer of *null* (Δ, 16 kb) vs. *flox* (10 kb) bands in Southern blots (data not shown). For our analysis in liver, we used mice that had 50% or more of *c-myc*-deleted alleles.

To determine whether *c-myc* gene deletion in liver affected parenchymal organization, we prepared hematoxylin/eosin (H&E)-stained sections from pIpC-injected 10-day-old and 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice. Liver sections showed disorganized parenchyma, hepatocytes with reduced cell area, and a greater incidence of pyknotic nuclei in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse hepatocytes compared with those of controls (Fig. 1*D*). These defects in liver organization correlated with impaired hepatic function in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice when compared with control mice (Fig. 1*G*). These results suggest that perinatal *c-myc* deletion in liver severely affects parenchymal organization and function in liver of homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice.

H&E-stained sections indicated that hepatocyte size might be altered in homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Overexpression of *c-myc* in B lymphocytes and hepatocytes increases cell size in mice (15, 17, 29). Accordingly, *Drosophila* Myc mutants have smaller cell size (16). In contrast, hepatocytes from mice harboring hypomorphic alleles of *c-myc* show normal cell size (20). To analyze whether *c-myc* affected hepatocyte size, hepatocytes were isolated from pIpC-injected homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice and analyzed by FACS. Forward scatter in FACS analysis showed a decrease in hepatocyte size in 10-day-old and 10-week-old *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice compared with control littermates (Fig. 2*A* and data not shown). We also observed that adult *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse hepatocytes had a smaller cell area than controls (Fig. 2*B*). Furthermore, adult *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ livers contained more hepatocytes per field (201.5 ± 22.8) than those of control littermates (135.8 ± 12.7) (Fig. 2*C*).

Acquisition of polyploidy by hepatocytes is an age-dependent differentiation process characteristic of liver growth. At birth, the majority of hepatocytes are diploid mononucleated cells that cycle normally. Subsequently, hepatocytes undergo a modified cell cycle without cytokinesis and generate binucleated polyploids cells (18). c-Myc regulates cell ploidy in human primary keratinocytes, mouse hepatocytes, and flies (19, 30–32). To determine whether the lack of c-Myc affected cell ploidy in liver, we isolated primary hepatocytes from pIpC-injected 10-week-old mice and stained them with propidium iodide to measure DNA content. Polyploid hepatocyte populations can be identified by the side-scatter parameter (Fig. 2*D*, gate R1). The polyploid population was reduced in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice compared with controls (Fig. 2 *D* and *E*). To see whether a decrease in the polyploid population correlated with a reduction in the number of binucleated cells in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice compared with control mice, we performed binucleated cell countings in H&E-stained liver sections. We observed a decrease in the number of binucleated hepatocytes in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice compared with control mice (Fig. 2*F*). These results indicate that c-Myc is necessary for normal acquisition of polyploidy in hepatocytes.

c-Myc is induced in proliferating cultured primary hepatocytes, and promotes progression from G_0/G_1 to S phase (8). To test whether c-Myc affects hepatocyte proliferation, we measured BrdUrd incorporation in liver sections of pIpC-injected 6- and 10-week-old mice. We observed a larger number of proliferating BrdUrd^{+} hepatocytes in the liver of 6-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice than in control mice (Fig. 3*A* and Table 1). To determine whether the *c-myc* gene in proliferating hepatocytes was intact, nondeleted (*flox/flox*) or deleted (*null/*

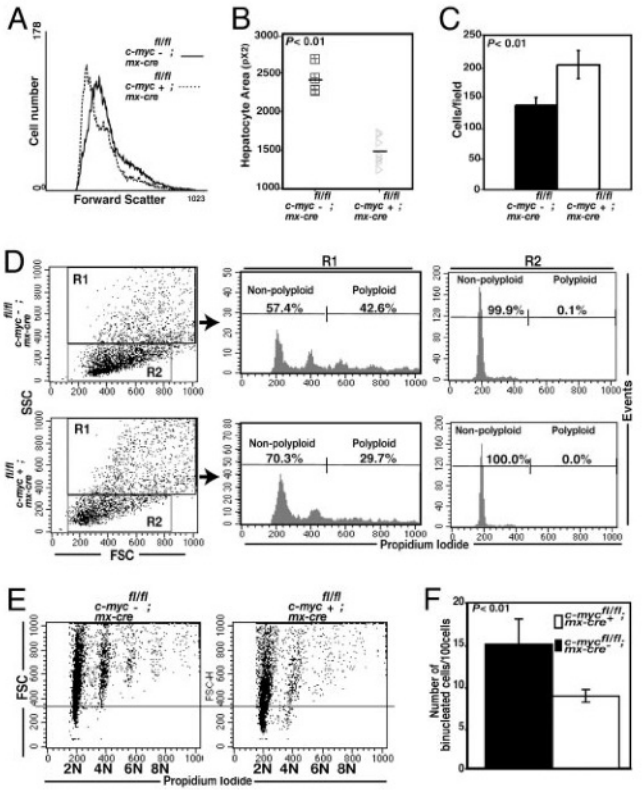

Fig. 2. Decreased cell size and ploidy in hepatocytes from *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$ *mice*. (*A*) Reduced hepatocyte size in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Hepatocytes from 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and *c-myc*$^{fl/fl}$;*mx-cre*$^{-}$ control mice were isolated and analyzed by FACS. A total of four mice of each genotype were analyzed. (*B*) Area of hepatocytes from H&E-stained liver sections of 10-week-old mice (IMAGEJ29 software). pX^2, square pixels. (*C*) Hepatocyte number per field (two fields per mouse) in liver sections from mice as in Fig. 1*D*. For cell area and hepatocytes per field, five control mice and nine homozygous mice were analyzed. (*D*) Decreased cell ploidy in hepatocytes from 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Side-scatter plot of whole populations is shown. R1 gate is used to discriminate polyploid cells; R2 gate contains nonpolyploid cells only. Numbers represent the percentage of cells in each gate within each plot. (*E*) Cell size vs. DNA content (propidium iodide). Hepatocytes from mice of indicated genotypes were isolated and analyzed by FACS. All mice were injected with pIpC as newborns. A line has been drawn to compare FSC values. Example representative of three independent experiments. (*F*) Reduced number of binucleated cells in liver from *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. The number of binucleated cells per field were counted (three fields per mouse), and the number was normalized by 100 cells per field. The graph shows the mean of four mice for each genotype. *P* value was determined by using Student's *t* test.

null), we performed a genomic PCR assay on single PCNA-positive cells isolated by laser (UV microdissection) from liver sections of homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. PCNA was used to select proliferating cells because genomic DNA from BrdUrd^{+} hepatocytes is not amplified by PCR (data not shown). Cells that gave a PCR product corresponding to the *c-myc*-deleted gene did not amplified a *flox* allele and vice versa. Therefore, this analysis identified cells with the *c-myc* gene deleted (Fig. 3*B*). These results show that hepatocytes are at least capable of entering the S phase of the cell cycle in the absence of *c-myc*. These analysis cannot rule out the possibility that BrdUrd^{+} or PCNA^{+} cells do not complete S phases. However, the fact that the ratio between deleted (null) and nondeleted (flox) hepatocytes in 10-day-old and 10-week-old mice is maintained supports the notion that c-Myc-deficient hepatocytes do

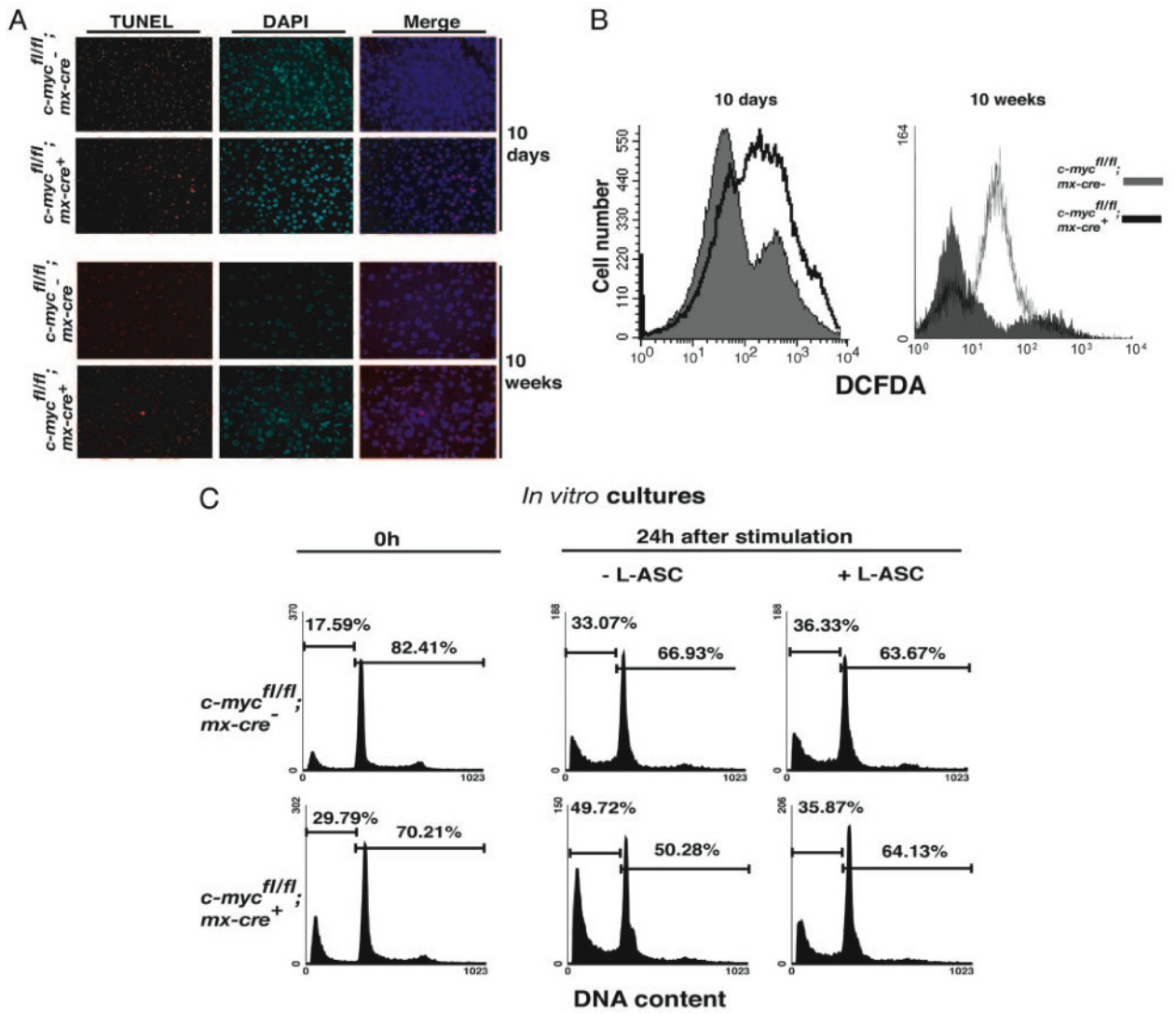

Fig. 4. Apoptosis in liver from *c-myc* $^{fl/fl}$;*mx-cre*$^{+}$. (*A*) TUNEL assays in liver sections from homozygous *c-myc* $^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice. (*B*) Increased free-radical content in homozygous *c-myc* $^{fl/fl}$;*mx-cre*$^{+}$ mouse liver. Hepatocytes from 10-day-old and 10-week-old homozygous and control mice were isolated and stained with the oxidation-sensitive probe DCFH-DA; a total of four mice of each genotype were analyzed. (*C*) Hepatocyte apoptosis is inhibited by the antioxidant L-ascorbic acid (L-Asc). Hepatocytes from *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice were isolated and cultured *in vitro* alone or with L-Asc for 24 h. Cells were harvested at times indicated, propidium iodide-stained to measure DNA content, and subG$_0$/G$_1$ peaks (dead cells) were analyzed by FACS. Experiment representative of three independent experiments. In all experiments, mice were injected with pIpC as newborns.

and 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice compared with controls (Fig. 4*A* and Table 2). Increase in free radicals content has been shown to precede apoptosis in hepatocytes and in mouse models of experimental liver injury (38, 39). In human fibroblasts, c-Myc can induce DNA damage by a mechanism mediated reactive oxygen species (40). To test whether the cell oxidative status in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse liver was affected, we analyzed primary hepatocytes from pIpC-induced mice with the oxidation-sensitive probe DCFH-DA. Ten-day-old and 10-week-old *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse hepatocytes showed a notable increase in DCFH-DA signal compared with control littermates (Fig. 4*B*).

To see whether increased apoptosis was mediated by free radicals, we treated activated primary hepatocytes from pIpC-injected 10-day-old *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice with the antioxidant ascorbic acid. We observed that primary hepatocytes from 10-day-old *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice were more sensitive to apoptosis during the isolation procedure than control hepatocytes (Fig. 4*C*, 0 h, and data not shown). Furthermore, we found inhibition of apoptosis in hepatocytes from *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice cultured in the presence of ascorbic acid to levels comparable with control cells (Fig. 4*C*). Apoptosis in hepatocytes from *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice is thus mediated by free radicals, and can be inhibited by ascorbic acid.

We show that perinatal deletion of *c-myc* severely affects liver organization and function in homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. In contrast to Trumpp *et al.* (20), we observe that *c-myc* can regulate cell size and number in liver. This difference could be explained by the intrinsic nature of the system used (hypomorphic vs. null *c-myc* alleles).

The capacity of c-Myc-deficient hepatocytes to proliferate might reflect the ability of hepatic stem cells to respond to the environmental factors present in each developmental stage (Fig. 3*A* and Table 1). It is possible that the effects observed in cell

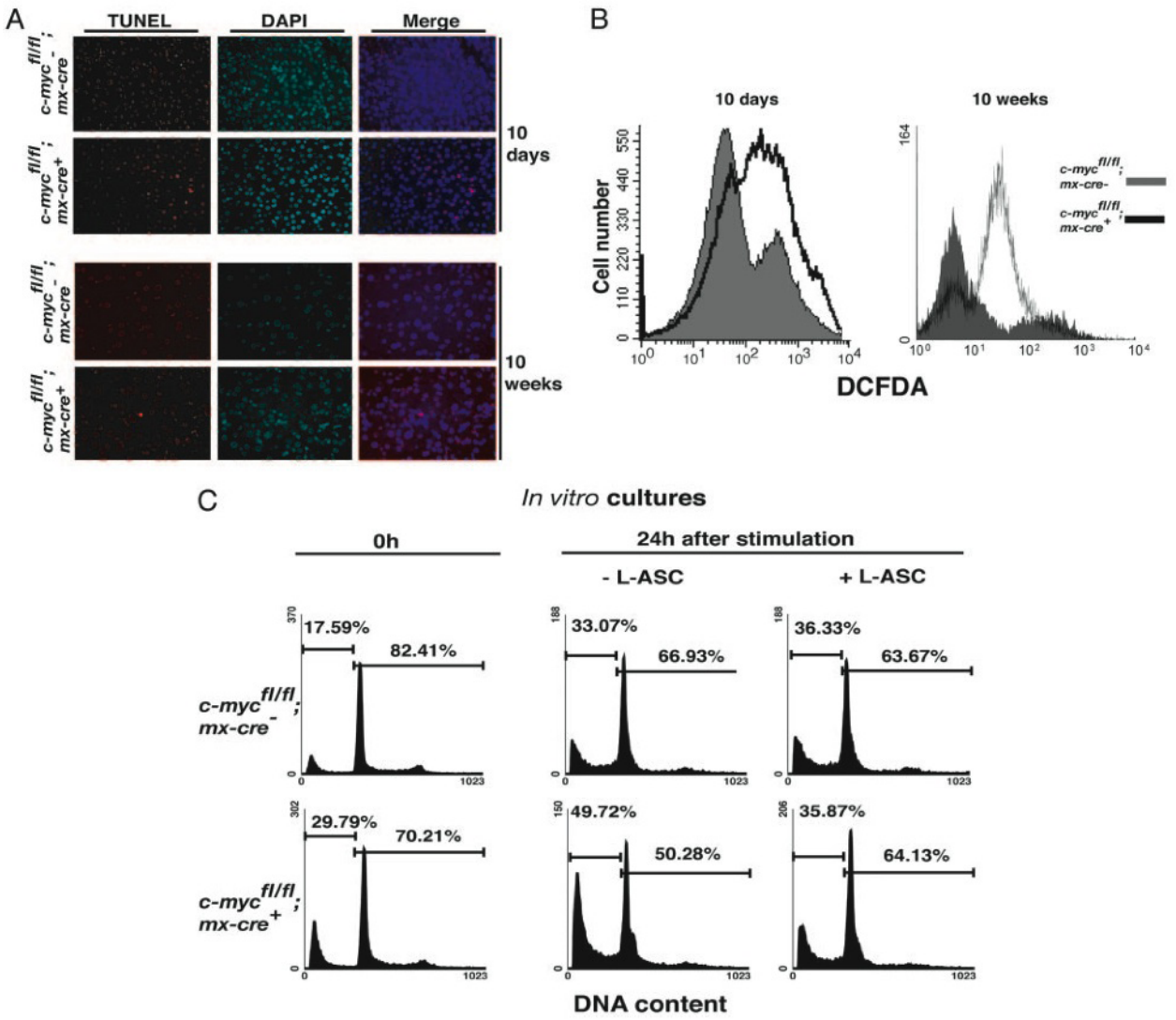

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Table 2. Quantification of TUNEL$^+$ hepatocytes

Mouse genotype	Cells per field	TUNEL$^+$, four fields	TUNEL$^+$, 100 cells
	10 days		
c-myc$^{fl/+}$;mx-cre$^-$	145	2	0.34
c-myc$^{fl/+}$;mx-cre$^-$	107	3	0.70
c-myc$^{fl/fl}$;mx-cre$^-$	121	1	0.21
c-myc$^{fl/fl}$;mx-cre$^+$	178	7	0.98
c-myc$^{fl/fl}$;mx-cre$^+$	169	6	0.89
c-myc$^{fl/fl}$;mx-cre$^+$	179	4	0.56
	10 weeks		
c-myc$^{fl/+}$;mx-cre$^-$	149	0	0
c-myc$^{fl/+}$;mx-cre$^-$	118	1	0.21
c-myc$^{fl/fl}$;mx-cre$^-$	145	0	0
c-myc$^{fl/fl}$;mx-cre$^-$	139	1	0.18
c-myc$^{fl/fl}$;mx-cre$^+$	210	3	0.36
c-myc$^{fl/fl}$;mx-cre$^+$	178	2	0.28
c-myc$^{fl/fl}$;mx-cre$^+$	230	1	0.12
c-myc$^{fl/fl}$;mx-cre$^+$	179	4	0.56
c-myc$^{fl/fl}$;mx-cre$^+$	223	0	0
c-myc$^{fl/fl}$;mx-cre$^+$	221	1	0.11

TUNEL$^+$ hepatocytes were counted from sections shown in Fig. 4*A*.

proliferation in livers of young and adult *c-myc$^{fl/fl}$;mx-cre$^+$* mice are directly related to the abundance of hepatic stem cells. Hepatic stem cells could respond to liver damage, decrease cell size, or surrounding cells with different c-Myc levels, by proliferating and differentiating in a c-Myc-independent manner in young mice. As mice get older, these hepatic stem cells either accumulate, lose the capacity to differentiate, or die by apoptosis. On this regard, c-Myc-deficient hematopoietic stem cells have been shown to proliferate and accumulate over time in the bone marrow of adult mice (41). Alternatively, liver cells seem to be very sensitive to changes in c-Myc expression. Mice overexpressing an inducible *c-myc* transgene in liver seem to expose the multipotency of hepatic stem cells to generate tumors, differentiate, or die, depending on the levels of c-Myc (42). Moreover, the response of liver cells to *c-myc* overexpression in liver is tightly linked to the developmental context (43). In the case of liver regeneration after PH, we observed that c-Myc is required for normal regeneration. This apparent contradiction might be explained by the time at which *c-myc* deletion is induced (newborn vs. adult). In adult mice, hepatocytes are quiescent (G_0) when *c-myc* deletion is induced and PH is performed; in this case, hepatocytes may require c-Myc to exit G_0. In contrast, after perinatal *c-myc* deletion, hepatocytes might not exit the cell cycle to enter the quiescence state (G_0) compared with control littermates (Fig. 3 *A* and *C*).

Homozygous *c-myc$^{fl/fl}$;mx-cre$^+$* mice showed reduced body size, and a slight increase in liver:body ratio compared with controls (Fig. 1 *E* and *F*). The mechanism(s) underlying the regulation of body and organ size are complex and beyond the scope of this paper. However, recent reports implicate *Drosophila* Myc in the regulation of organ size by cell competition in *Drosophila* imaginal discs (21, 22). We cannot rule out that the apoptosis observed in the liver of *c-myc$^{fl/fl}$;mx-cre$^+$* mice is due to wt cells inducing cell death of c-Myc-deficient cells as observed in *Drosophila* (21, 22). However, the presence of c-Myc-deficient hepatocytes in those mice that harbor extensive *c-myc* deletion 2 months after induction does not favor this possibility (Fig. 1*B*). Further studies are required that directly address these questions. On this regard, *c-myc$^{fl/fl}$;mx-cre$^+$* mice should be a useful model.

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